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SECTION MEETINGS

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Isolation of a Feline Virus Associated with Intranuclear Inclusion Bodies. (23783)

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During the summer of 1957, an outbreak of an upper respiratory disease occurred in a group of 5-10-week-old kittens. It was acute, infectious and characterized by conjunctivitis with lacrimation, and nasal discharge accompanied by coughing and sneezing. A viral agent, cytopathogenic for feline kidney cells in tissue culture, was isolated at the time the kittens first exhibited signs of sickness. This paper is a report on the isolation of this agent with a description of the inclusion bodies in tissue cultures and in the cat.

Materials and methods. Tissue cultures of feline kidney cells were prepared in tubes by the trypsin digestion method described by Madin(1). The cells were grown in a nutrient medium consisting of 0.5% lactalbumin hydrolysate in modified Hanks solution with 10% lamb serum. The maintenance fluid used at the time of inoculation was lactalbumin hydrolysate with the addition of 5% lamb serum. Streptomycin (0.5 mg/ml), penicillin (500 u/ml) and mycostatin (100 u/ml) were incorporated in all media. *Isolation technics*: Primary isolation of the agent was made by swabbing the throat and conjunctiva with cotton swabs which were then placed in feline kidney tissue cultures. After 3 hours incubation at 35°C the nutrient fluid was removed and the monolayer rinsed twice and covered with 1 ml of medium prior to additional incubation. Titrations of this agent were made by inoculation of 0.1 ml of decimal dilutions into each of 4 tubes of feline kidney tissue culture. The 50% cytopathogenic end point ($TCID_{50}$) was calculated by the method of Reed and Muench(2). Replicate titrations of virus-containing fluid of various tissue culture passages showed $TCID_{50}$ of 10^{-6} to $10^{-6.6}$ per 0.1 ml. Serial 2-fold dilutions of the inactivated sera were mixed with equal volumes of tissue culture

fluid containing approximately 200 $TCID_{50}$/0.1 of the virus. The serum-virus mixtures were incubated at room temperature for 1 hour. Then 0.1 ml of each mixture was inoculated into each of 4 tubes of feline kidney tissue culture. The presence of neutralizing antibodies was indicated by the absence of cytopathogenic changes. Both tissue cultures and necropsy material were fixed in Bouin's fluid and stained with either hematoxylin and eosin or Giemsa. Histological preparations of tissue culture were made employing either coverslips or the collodion technic(3).

Results. Small foci of degeneration, characterized by rounding of the cells, were first observed after 24 hours of incubation (Fig. 1 and 2). At this time intranuclear inclusion bodies were demonstrated in stained preparations (Fig. 3). These inclusions, when well developed, were homogeneous, acidophilic, usually spherical or oval and separated from the nuclear membrane by a clear halo. The

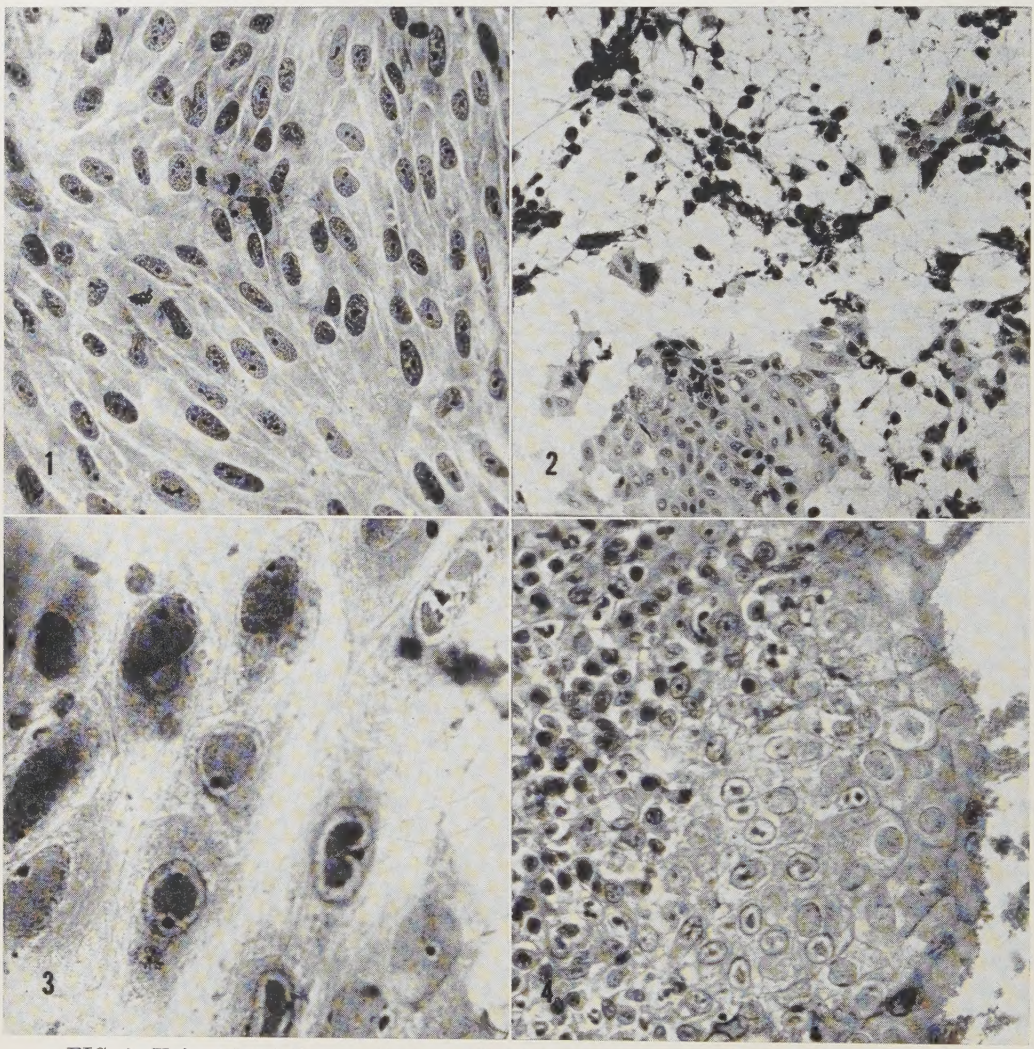

FIG. 1. Uninoculated tissue culture of feline kidney. Giemsa ×265, AFIP-MIS 57-14274.
FIG. 2. Cytopathogenic changes in tissue culture infected with feline virus. H & E ×100, AFIP-MIS 57-14275.
FIG. 3. Intranuclear inclusions in culture of feline kidney with feline virus. H & E ×755, AFIP-MIS 57-14287.
FIG. 4. Intranuclear inclusions in epithelial cells of tonsil in the domestic cat. H & E ×400, AFIP-MIS 57-14343.

nucleoli usually persisted at the edge of the inclusion. These inclusions still were observed in the 12th and last tissue culture passage made to date. All attempts to culture the agent on bacteriological and PPLO media have been unsuccessful. The agent was given the laboratory designation of C-27 virus.

Four kittens were inoculated intranasally with 0.5 ml of tissue culture fluid from the 3rd passage. One uninoculated kitten was placed in an adjoining room as a control. Pyrexia, lacrimation and nasal discharge with coughing and sneezing developed in the 4 kittens, 1 to 3 days after inoculation. The control kitten remained normal throughout the experiment.

Both preinoculation and postinoculation sera from the experimental kittens were tested for specific neutralizing antibodies against the virus. All preinoculation sera failed to prevent cytopathogenic changes. No neutralizing antibody was demonstrated in the postinoculation sera from the 2 kittens sacrificed at 48 and 72 hours. In the animal sacrificed 17 days after inoculation the appearance of cytopathogenic changes was delayed. However, the serum from the remaining kitten, drawn 21 days after inoculation, neutralized the virus in a 1:4 dilution. Serological studies are being continued to confirm this immune response to the agent.

Tissues from the 2 kittens sacrificed at 48 hours and 72 hours were fixed in Bouin's fluid and sections were stained with hematoxylin and eosin. Intranuclear inclusions similar to those in tissue culture were demonstrated in the epithelium of the turbinate, tonsil, nictitating membrane and trachea (Fig. 4 and 5).

In the experimental kittens inclusions were most numerous in the pseudostratified columnar and columnar epithelium of the upper respiratory tract. The apparent sequence of inclusion development, as suggested by the stages in cell variation from normal to those with well developed inclusions, is as follows: The normal columnar epithelial cell stained with hematoxylin and eosin has an acidophilic cytoplasm and basophilic nucleus with a distinct nucleolus and fine, evenly distributed chromatin. Affected cells show margination of the nucleolus

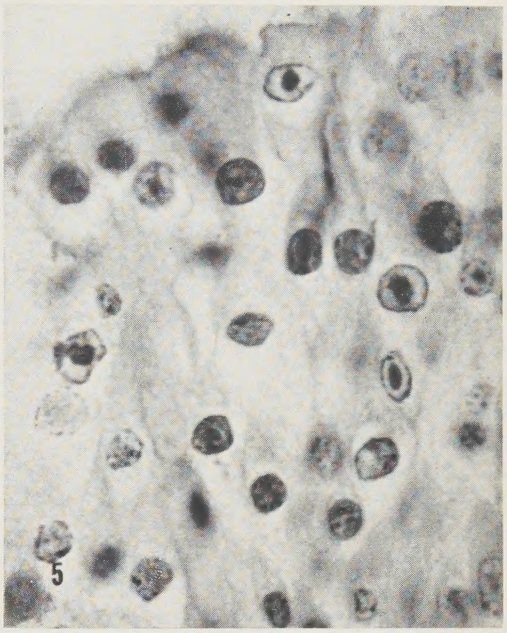

FIG. 5. Intranuclear inclusion in epithelial cells of turbinates in the domestic cat. H&E ×850, AFIP-MIS 57-14339.

and the chromatin, with eventual disappearance of the nucleolus. The center of the nucleus becomes eosinophilic. The homogeneous acidophilic material filling the nucleus contracts, leaving a clear halo around the now spherical inclusion. Well formed inclusions occupy from one-third to one-half of the otherwise clear space within the often shrunken but intact nuclear membrane. Presence of the intranuclear inclusions is usually associated with degenerative changes in the cytoplasm. The cytoplasm becomes pale, later forming a clear zone around one side of the nucleus. In addition to the usual clearing near the nucleus, the cytoplasm may become ballooned, granular and necrotic, losing its distinct outline with consequent disruption of the superficial mucosa. When this occurs localized infiltration of polymorphonuclear cells with submucosal congestion and edema may be seen.

The virus in tissue culture fluid (pH 7.4) survived storage at –60°C for at least 3 months. It was successfully lyophilized.

To determine something of the host range of the virus, 5 day embryonated eggs, weanling mice and rabbits were inoculated. The

agent was not recovered from eggs 5 days after inoculation via the chorioallantoic membrane. No deaths or illness occurred in mice inoculated intracranially or in rabbits inoculated by corneal scarification, both observed for the following 21 days.

Discussion. The 3 feline viruses to which this agent (C-27) was compared were those of feline pneumonitis(4), and panleucopenia (5,6) and the kidney cell degenerating virus (KCD)(7). Comparison was made of clinical symptoms, histopathologic features of necropsy material and the cultural characteristics of the virus in a tissue culture host.

The C-27 virus involves predominantly the upper respiratory system, producing symptoms similar to those of feline pneumonitis. In infections with C-27 virus intranuclear inclusions are observed in epithelial cells of the upper respiratory tract and in tissue cultures; intranuclear inclusions are not known to occur in feline pneumonitis. Furthermore, feline pneumonitis virus in suitable hosts forms elementary bodies(8) characteristic of the psittacosis-lymphogranuloma group and it has not been propagated in tissue culture systems.

Leucopenia, thirst and enteritis, recognized symptoms of feline panleucopenia, are not observed in the disease described here. Intranuclear inclusions in panleucopenia have been reported(6,9), but only in the intestinal mucosa, and recent study(10) indicates that they are not demonstrable consistently.

Although Fastier's(7) KCD virus produced cytopathogenic changes in feline kidney cell cultures, it did not form inclusions or elementary bodies, nor did it cause clinical disease in cats. Fastier considered it an orphan virus.

The evidence presented suggests that C-27 is a previously unrecognized viral agent pathogenic to the domestic cat.

Additional studies are in progress to characterize and identify the virus and the disease more fully.

Summary. 1) A viral agent cytopathogenic for feline kidney tissue culture was isolated from a cat with a respiratory infection. It has been shown to be capable of reproducing the illness in susceptible kittens with the development of specific neutralizing antibodies. Intranuclear inclusions have been demonstrated in both tissue culture preparations and kittens in which the disease was reproduced. 2) The agent has survived lyophilization and storage in tissue culture fluid for at least 3 months at –60°C.

The authors wish to acknowledge the technical assistance of Edward W. Despeaux in conducting this work.

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Further Observations on Effect on Plasma 17-OH-Corticosteroids in the Dog of Derivatives of 2,2-Bis-(p-Chlorophenyl)-1,1-Dichloroethane (DDD, TDE).* (23784)

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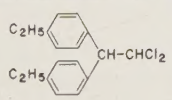
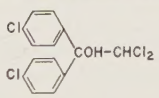
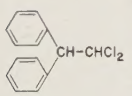
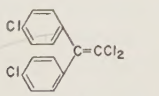
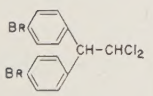
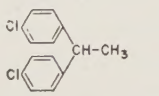
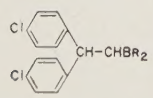
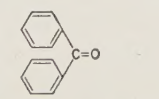
Production of adrenal cortical atrophy in dogs by 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane (DDD, TDE) was first observed by Nelson and Woodard(1). Larson *et al.*(2) demonstrated that while DDD and certain of its derivatives produce adrenal cortical atrophy, still other derivatives cause hyperplasia or hypertrophy. The production of adrenal cortical atrophy is dependent upon the presence of the intact 2,2-diphenyl-1,1-dichloroethane structure. Alteration of the number of chlorines on the 1-position of the ethane abolishes the ability to produce adrenal cortical atrophy. Hydroxylation at the 2-position or desaturation to the ethylene analogue forms compounds which produce adrenal cortical hypertrophy. In an investigation of the effect of DDD and compound I (Table I) on the level of plasma 17-hydroxycorticosteroids (corticoids) in dogs, Cobey, Taliaferro and Haag(3) found that adrenal cortical atrophy obtained with these compounds was accompanied by decreased response to ACTH as measured by changes of plasma corticoids. However, a similar decrease was found using compound V which has been shown(2) to produce adrenal cortical hypertrophy.

TABLE I. Structural Formulae of the 8 DDD Derivatives Studied. Compound I is referred to as Perthane® and Compound V as FW-152.

REFERENCE NUMBER	COMPOUND	REFERENCE NUMBER	COMPOUND
I		V	
II		VI	
III		VII	
IV		VIII	

The purpose of the present investigation was primarily to obtain further information on the effect on corticoid secretion of DDD derivatives, incluing several which have been previously studied and certain additional ones. In some instances, the histopathological effects on the adrenal cortex were also determined.

Procedure. Mongrel dogs of both sexes weighing from 15 kg to 30 kg were used. With the exception of Compound I, the DDD derivatives† were dissolved in corn oil and placed in gelatin capsules for oral administration. Compound I was used for long-term feeding studies in which it was administered thoroughly mixed with finely ground Purina Dog Chow Kibbled Meal. Routinely, 2 hours before blood samples were taken, 20 U.S.P. units of ACTH‡ was administered intravenously so as to determine the secretory responsiveness of the adrenal cortex(3). Plasma 17-hydroxycorticoids§ were determined by the method of Nelson and Samuels(4).

Results. The derivatives studied are shown in Table I, and, with the exception of compounds VII and VIII, the results obtained are shown in Fig. 1-3.

Compound I (Perthane®) was given to 2 dogs in the diet at levels of 500 and 1000 p.p.m. (parts per million) rather than in capsule form as we had done previously(3). Within 2 weeks at each level a marked diminution was noted in ability of the adrenal

* This work was supported by Grant from the Nat. Heart Inst., N.I.H., U. S. Public Health Service.

† Kindly furnished through the courtesy of Rohm and Haas Co., Philadelphia.

‡ Armour's Corticotrophin (ACTH), 25 I.U./vial.

§ Standard 17-hydroxycorticosteroid was kindly supplied by Dr. H. Molitor, Merck Institute for Therapeutic Research, Rahway, N. J.

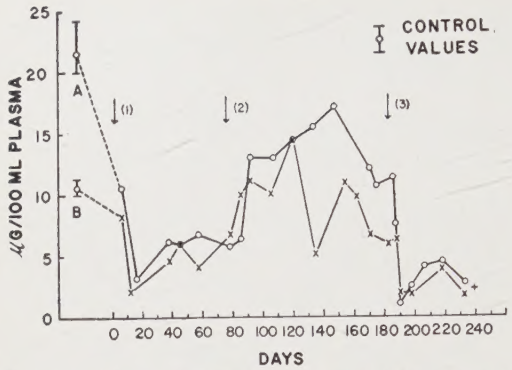


FIG. 1. Effect of Compound I in 2 dogs (A and B) on level of plasma 17-hydroxycorticosteroids 2 hr after inj. of ACTH. Arrows indicate addition of Compound I to the diet as follows: 500 p.p.m. level started at (1) and discontinued at (2); 1000 p.p.m. started at (3). Animals sacrificed at ×.

cortex to respond to ACTH as measured by levels of plasma corticoids (Fig. 1). The plasma corticoids showed some increase after the initial drop, but adrenal responsiveness was maintained at a definitely lower than normal level as long as treatment was continued. The decrease in corticoids while the dogs were on the diet of 1000 p.p.m. was essentially the same as that noted while on the diet of 500 p.p.m. In one animal (Dog A)

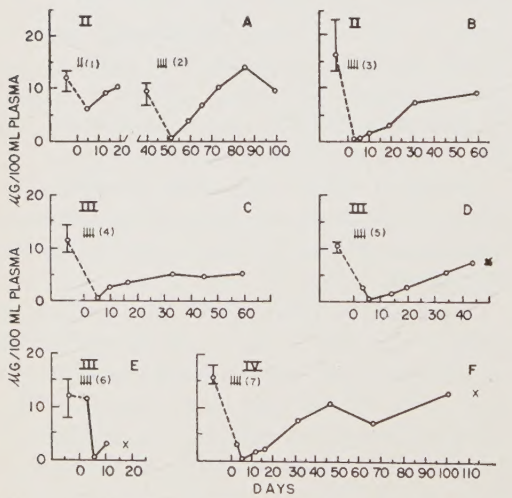


FIG. 2. Effect of Compounds II, III and IV in dogs on level of plasma 17-hydroxycorticosteroids 2 hr after inj. of ACTH. ♀ represents control values; ↓ indicates oral administration of compounds in corn oil solution as follows: (1) and (6) 100 mg/kg/day; (2), (3), (4), (5) 200 mg/kg/day. Animals sacrificed at ×.

the plasma corticoid values did not return to the normal level after compound I was discontinued. In this connection it might be pointed out that with this series of compounds it was frequently observed that after completion of treatment during which a marked decrease in plasma corticoid output was obtained, the plasma levels would not return to their former value for periods of up to several months. Larson *et al.*(2) as well as the authors(3) have previously found that this compound produces adrenal cortical atrophy in dogs. Similarly, in the present study Dog

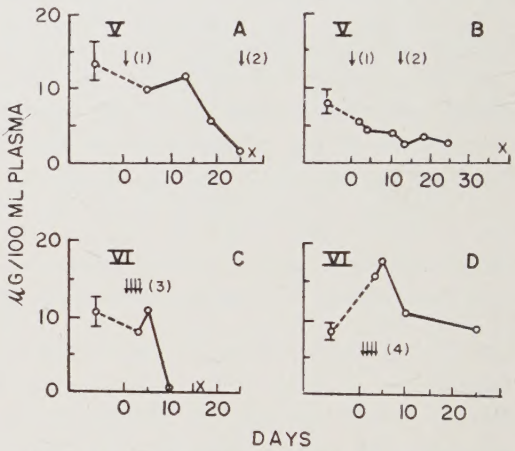


FIG. 3. Effect of Compounds V and VI in dogs on the level of plasma 17-hydroxycorticosteroids 2 hr after inj. of ACTH. ♀ represents control values; ↓ indicates oral administration of compounds in corn oil as follows: (1) 50 mg/kg/day started and stopped at (2); (3) 200 mg/kg/day; (4) 100 mg/kg/day. Animals died or were sacrificed at ×.

A showed moderate atrophy of the zona fasciculata at autopsy and Dog B, marked atrophy of the zona fasciculata.‖

Compound II, different from Compound I in that it has no para-phenyl substitution, markedly decreased the ability of the adrenal cortex to secrete corticoids in the 2 dogs studied (Fig. 2, II A, B). The fall of plasma corticoids obtained with this compound is similar to that previously demonstrated for

‖ The authors wish to thank Dr. G. R. Hennigar for making the histopathological interpretations and to gratefully acknowledge the technical assistance of Angela Cahn and Jean Sadler in doing the plasma corticoid determinations.

Compound I and DDD(3), but recovery from its effects was somewhat more rapid than from Compound I. This is in line with the observation of Larson *et al.*(2) that Compound I produces a greater degree of atrophy than Compound II. As shown with *Compound III,* the substitution of bromine for chlorine on the para-phenyl positions of the DDD molecule did not materially alter its effects on the adrenal. This compound caused a decrease in adrenal responsiveness (Fig. 2, III C, D, E) comparable to that observed with Compounds I and II. Dogs D and E were sacrificed for autopsy studies. The adrenal of Dog D showed moderate atrophy of both the zona reticularis and zona fasciculata, while the adrenal of Dog E was reported as showing "atrophy of the fasciculata."

Another bromine derivative, *Compound IV,* in which the aliphatic chlorine of DDD was replaced by bromine, caused a fall in the plasma corticoids from 16 to 0 μg per 100 ml plasma following treatment (Fig. 2, IV F) in the one dog studied. Insufficient material precluded further experiments. When this dog was sacrificed almost 3 months after dosing, no evidence of adrenal damage was found on histopathological examination. In this instance the adrenal cortex had apparently been physiologically blocked as far as elaboration of corticoids was concerned without showing any evidence, of an irreversible character at least, of anatomical alterations.

Compound V (FW-152) is DDD hydroxylated on the 2-position of the ethane moiety. It caused a depression of adrenal cortical function (Fig. 3, V A, B), in the 2 dogs studied. This is in confirmation of a preliminary experiment already reported(3). Because of its relative toxicity as compared to other members of the series, Compound V was given at a dosage level of 50 mg/kg body weight. Even at this low dosage the 2 dogs developed elevated plasma bilirubin levels with total values of 2.9 and 1.6 mg per 100 ml after 18 days, and each of the animals lost 3 kg of weight during the experiment. Larson *et al.*(2) had found that this compound produced moderate to marked adrenal cortical hypertrophy. At autopsy the 2 dogs in the present study showed deeply jaundiced tissues. Unfortunately autolytic changes made histopathological examination in one dog impossible, and in the case of the other, the specimen was lost.

When Compound VI, an ethylene analogue of DDD, was administered to one dog at a level of 200 mg/kg body weight for 4 days, the plasma corticoids fell to zero at the tenth day and the animal died on the sixteenth day (Fig. 3, VI C). In the next experiment the dosage was decreased to 100 mg/kg for 4 days; at this dosage the animal became very ill, but recovered. The plasma corticoids of this dog *rose* from 8 to 18 μg per 100 ml plasma on the fifth day and then gradually returned to normal level (Fig. 3, VI D). Due to our inability to obtain more of this compound it was not possible to repeat these experiments. Whether this apparent reversal in corticoid response following the smaller dose of Compound VI was due simply to a natural periodic variation in this dog the adrenals of which were unaffected by this dose of Compound VI, to a synergism between Compound VI at this dose level and ACTH, or to a combination of these and/or other factors, is not known. However, with reference to the possibility that this might be an instance of natural variation, it should be pointed out that both our present control experiment (see below) and past experiences(3) indicate that this is quite unlikely. Larson *et al.*(2) had previously found that this compound produces moderate hypertrophy of the adrenal cortex involving both the zona glomerulosa and zona fasciculata.

Compounds V and VI were of special interest because, in the absence of demonstrable pathology in other organs, Larson *et al.*(2) found marked or moderate adrenocortical hypertrophy in 3 dogs which survived for 2 or 3 weeks of daily administration of Compound V, and moderate adrenocortical hypertrophy in 2 dogs which survived 2 weeks of daily administration of Compound VI. Furthermore, in one of the 2 dogs treated with Compound V by Cobey *et al.*(3), plasma 17-hydroxycorticosteroid levels fell from 14 to 2 μg % after treatment. It was therefore considered probable that Compounds V and VI blocked steroidogenesis in, or release of steroids from an

hypertrophied adrenal cortex. The fall in corticoids following ACTH (Fig. 3, A, B, C) is consistent with this thesis. However, development of jaundice in the present studies with Compound V, and the lack of pathological study of the 4 animals receiving Compounds V and VI prevents acceptance of the attractive conclusion that the hypertrophied gland is a blocked gland incapable of responding to ACTH. Although not proven, it can be suggested, nevertheless, that Compounds V and VI do act in this manner, that is, cause hypertrophy of the adrenal cortex while blocking steroidogenesis or release of corticoids. Another compound known to have such properties is amphenone, a substance which produces hypertrophy with diminution of function. In rats amphenone produces marked accumulation of lipoid and hyperplasia of the adrenocortical cells(5). In dogs it causes hypertrophy and lipoid accumulation in the zona fasciculata and zona reticularis which is accompanied by diminution in adrenal venous blood content of 17-hydroxycorticosteroids and failure to respond to ACTH (6). From the revised formula of amphenone (7) it appears that the DDD derivatives and amphenone have structural similarities.

Compound VII represents DDD in which the chlorine on the 1-position is replaced with hydrogen. In the one dog studied, its administration, in a dose of 200 mg/kg daily for 4 days, produced no changes in the plasma corticoid level. Lack of material prevented further testing. This compound was shown by Larson *et al.*(2) to cause moderate adrenal cortical hyperplasia in the one dog used.

Compound VIII, benzophenone, was investigated because of the possibility that it might occur in the body as a metabolite of DDD. It produced no significant change in the plasma corticoids in 3 dogs given 200 mg/kg per day for 4 days.

One dog was maintained as a control for 23 weeks and determinations of the plasma corticoids were made at intervals (paired with an experimental dog) following the procedure used for the experimental animals. Capsules containing corn oil were given in the same manner (daily, for 4-day periods) in which the DDD derivatives were administered. Sixteen determinations were made and no significant variations were observed in the plasma corticoid levels in this control dog receiving corn oil.

Summary. 1) The effect of 8 derivatives of 2,2-bis-(p-chlorophenyl) - 1,1-dichloroethane (DDD, TDE) on responsiveness of the adrenal cortex to ACTH, as determined by changes in plasma 17-hydroxycorticoids, has been studied in dogs. The following derivatives decreased the corticoid response: those with para-phenyl modifications; bromine substitution of the aliphatic chlorines; hydroxylation on the 2-position of the ethane moiety. No change in response was noted with a derivative in which hydrogen replaced chlorine in the 1-position of the ethane moiety, and with benzophenone, a possible DDD metabolite. Somewhat equivocal results were obtained with an ethylene analogue. 2) The relationship between these biochemical findings and the histopathologic effects recorded for this series of compounds is discussed.

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Antagonistic Effects of Cortisone and Growth-Hormone on the Developing Chick Embryo.* (23785)

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In a study of effects of various agents on the fate of grafts to the chorio-allantoic membrane of the chick embryo, it was noted that cortisone acetate when applied to the chorio-allantoic membrane at maximum dose permitting the embryo to survive, resulted in a striking inhibition of embryonic growth and development. These observations corresponded to the findings of Karnofsky *et al.*(4), who fully described the specific "cortisone effect" which, in addition to growth inhibiting action, also involved characteristic developmental modifications. As these effects were produced by relatively small doses of the steroid and as the effective dose of cortisone (1 mg/egg) resulted in very high mortality of the treated embryos, we searched for an agent to alleviate the deleterious systemic action of the steroid and to increase viability of the treated embryos, and possibly tolerance of larger doses of cortisone. On grounds of the type of certain histological changes in tissues of cortisone treated embryos the attempt was made to counteract the action of cortisone by administration of growth hormone (somatotrophic hormone, STH). It has been shown that in cortisone-treated chick embryos there is an excessive excretion of sodium chloride and reducing sugars into the allantoic fluid (Danowski, *et al.*(2). It could be assumed that the negative balance of these substances in the embryo might play a part in producing the symptoms of the "cortisone effect." Therefore experiments were also made in which saline with glucose, *i.e.* Tyrode's solution, was administered to cortisone treated embryos.

Material and methods. Fertile white Leghorn eggs were incubated at 38°C. Injections of the various agents were made under sterile conditions onto the chorio-allantoic membrane through a small window cut out in the shell directly over the membrane. After injection, the opening was covered by a coverslip and sealed with paraffin. Three series of injections were run concurrently: a) one in which embryos of 8 days of incubation were injected with a single dose of 1 mg of cortisone, b) a second in which embryos injected with the same dose of cortisone at the same age were, in addition, treated with growth hormone. In this series the embryos were injected 24 hours previous to cortisone treatment with 250 γ of growth hormone dissolved in Tyrode's solution and these injections were repeated afterwards daily; and c) a third series in which embryos were treated in a similar way as in series b) except that Tyrode's solution was substituted for growth hormone solution.‡ The embryos which died before the 13th day of incubation were discarded, even though the "cortisone effect" could be first clearly seen at 10th to 11th day. The embryos surviving to the 15th day were sacrificed, weighed, measured and examined.

Results. Effects of cortisone on chick embryo. The effect of cortisone on the developing embryo expressed itself in the high mor-

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† This work was done during tenure of Research Fellowship granted by Ministry of Education, Government of Israel.

‡ Growth hormone, in lyophilized form, was kindly supplied by Armour Laboratories, Chicago, through the courtesy of Dr. J. Bunding. Cortisone (11-dehydro-17-hydroxy-corticosterone acetate, "Cortone," Merck & Co.) was available in aqueous suspension, preserved in 1.5% benzyl alcohol necessary to produce appreciable injury to the chick embryo was 10 mg (Karnofsky(4)), whereas the amount of only 0.5 mg was injected with 1 mg of cortisone. It was found that Tyrode's solution when injected daily to otherwise untreated eggs did not affect detectably the development and growth of the embryos.

tality, in inhibition of growth and various developmental changes of surviving embryos. Our results corroborate in general the findings of Karnofsky *et al.*(4). The mortality of embryos injected with 1 mg of cortisone amounted to 40% (Table I); surviving em-

TABLE I. Mortality in Chick Embryos Injected with Cortisone and STH. Dose of cortisone, 1 mg per egg; age at injection, 8 days.

Additional agent	No. of eggs inj./No. dying (% mortality)
None	20/8 (40%)
Tyrode	27/6 (22)
STH	32/5 (17)

bryos were very small and showed characteristic curling of the body, the beak being pressed tightly against the left foot; feather formation was markedly deficient, the body being pale and somewhat edematous, the eyes were protruded and disproportionally large (Fig. 1). All embryos had exteriorized viscera, while the heart was greatly enlarged, the liver yellow and relatively very small, especially its left lobe. Embryonic membranes were deficient in development.

Administration of Tyrode's solution to the cortisone-injected embryos brought about a small but significant drop in mortality rate of the embryos (as shown in Table I). The probable explanation for this result will be discussed later This increase in survival rate was, however, not accompanied by a significant increase in growth and failed to prevent occurrence of the characteristic cortisone syndrome in the embryo.

FIG. 1.

Action of growth hormone on cortisone-treated embryos. When injected as described above, growth hormone showed a marked counteracting action on the effects brought about by cortisone in the chick embryo. The high mortality was lowered to 17%, reaching approximately the level of that occurring spontaneously during incubation. The slightly higher than usual percentage of mortality of treated embryos (about 2%) may probably be attributed to trauma of the injection procedure. Since the high mortality rate, caused obviously by cortisone, was also lessened, although in a much lower degree, by administration of saline with glucose, the most striking effects of growth hormone are those concerning growth and development of the embryo and the embryonic membranes. Embryos treated with cortisone and growth hormone showed a marked increase in body length and weight, when compared with those treated with cortisone only (Table II). Organs and various regions of the body attained almost normal proportions, the neck being relatively long, the eyes of usual size and shape. The occipital region of the head showed the usual curvature, the legs and wings were properly developed; the liver was of normal shape and of usual size. In many cases the abdominal wall was closed over the viscera as usual at this age. On the pteryle regions of the skin, which was pinkish in colour, feather papillae were formed and growing feathers were distinct (Fig. 1).

Concerning the embryonic membranes the yolk-sac seemed normally developed, enclosing the yolk to the usual degree. The chorio-allantoic membrane, however, showed a specific, repeatedly occurring thickening and a parchment-like texture, especially noticeable at the site of injections and around it. This thickening of the chorio-allantoic membrane,

TABLE II. Weight and Length of Chick Embryos Treated with Cortisone and STH.

	No. of embryos	Avg wt at 15 days, g	Avg length at 15 days, cm
Cortisone	15	3.1	4.3
" + Tyrode	24	3.6	4.4
" + STH	30	5.6	6.2

which in some cases attained the diameter of 2-3 mm interfered with the spatial position of the embryo in the egg and probably also with the functions of the extra-embryonic blood system.

Some other modifications caused by cortisone were not alleviated by the growth hormone. Embryos treated with both hormones retained the curling of the body, with the foot pressed against the head, and in some cases the viscera remained exteriorized and the heart was disproportionally enlarged.

Discussion. The present experiments demonstrate that the deleterious effects of cortisone on growth, development and survival of the chick embryo can be partially counteracted by simultaneous administration of growth hormone. This counteraction is expressed not only in an increased rate of survival, but also in the amelioration of many of the typical symptoms of cortisone treatment (Karnofsky *et al.*(4)), resulting in formation of nearly normal embryos. It seems, therefore, safe to suggest that the actions of these 2 hormones might be antagonistic in many respects.

The mode of action of cortisone in the embryo is unquestionably complex and comprises mainly two types of effects: a general inhibition of development of the embryo, expressed by retardation of growth and increase in body weight and length; and secondly, a disturbance of various physiological and metabolic processes as indicated by enlargement of heart, fatty degeneration of liver, changes in elimination of electrolytes and sugar. All these effects contribute, in extreme cases, to an increase in mortality of the embryos. In addition a local inhibitory effect of cortisone at site of its application to the chorio-allantoic membrane is observable in early embryos, where it prevents normal growth of the membranes.

The effect of cortisone on growth and development of embryos may, at least partly, be attributed to the known growth inhibiting action of this steroid on actively proliferating tissues, *e.g.* granulation tissue, newly forming cartilage and bone (Ragan *et al.*(6); Ragan (7)) and various tumors (Ingle *et al.*(3)); Stock(10) and others). This action affects principally the connective tissues and their derivatives, causing a delay in the formation of all elements of these tissues.

The general growth-stimulating effect of growth hormone (STH) on intact and hypophysectomized animals can also be traced, at least in part, to the action of this hormone upon connective tissues and their derivatives. Thus, for example, in juvenile rats growth hormone promotes proliferation of epiphyseal cartilages and stimulates osteogenesis (Simpson *et al.*(9)), while in full grown animals increased growth hormone stimulation results in appositional bone growth and in proliferation of soft tissues (acromegaly, Selye(8)).

The distinctly opposite effects of the 2 hormones under consideration suggest strongly that their actions are actually antagonistic. Recent experiments have indeed shown that growth hormone counteracted almost completely the inhibiting effects of cortisone upon formation of granulation tissues (Taubenhaus *et al.*(11)). The present observations on the developing embryos demonstrate that the antagonism is effective also at the level of systemic actions of these hormones, thus supporting the conclusion that growth hormone may act as a true antagonist to cortisone.

The development of feathers in cortisone and growth hormone treated embryos seems of interest. Cortisone almost completely inhibits formation of feather follicles, acting apparently selectively on dermal papillae. Embryos treated simultaneously with cortisone and growth hormone show considerably improved feather formation and consequently a richer plumage. This may be interpreted as due to abolition of the inhibiting effect of cortisone upon development of dermal papilla, the latter subsequently stimulating growth and differentiation of the ectodermal elements of the feather (Lillie & Hsi Wang(5)).

There remains to be mentioned the effect of Tyrode's solution on cortisone-treated embryos. The changes in chemical composition of the allantoic fluid in the cortisone treated embryos described by Danowski *et al.*(2) may provide a clue to the mechanism of influence of Tyrode's solution on survival of embryos.

In cortisone-treated embryos a marked increase in sodium, chloride and sugar (reduc-

ing substance) concentration in the allantoic fluid can be observed, while the potassium level is noticeably lowered. These alternations in electrolyte and sugar elimination may have some bearing upon the high mortality rate of such embryos. Addition of Tyrode's solution, consisting of physiological saline and glucose, to cortisone injected embryos raises slightly their survival rate. The results indicate a compensation therapy effect of the solution on embryos under conditions of increased sodium, chloride and sugar excretion.

Summary. 1) Single injections of 1 mg/egg of cortisone on the chorio-allantoic membrane of chick embryos produce a striking and specific syndrome: mortality of the embryos is highly increased, growth of surviving embryos is markedly retarded and characteristic developmental modifications are produced; electrolytes and sugar metabolisms are disturbed. 2) Simultaneous administration of Tyrode's solution, consisting of saline and glucose, to such embryos brings about a slight rise in survival rate of embryos, owing probably to its compensation therapy effect upon loss of sodium chloride and sugar (reducing substance). 3) Treatment of cortisone-injected embryos with growth hormone (STH) results in striking alleviation of most of the cortisone caused changes. Mortality rate is lowered almost to the level of that occurring spontaneously during incubation; inhibition of growth as well as many deleterious modifications are successfully counteracted. 4) Various aspects of the interaction of hormones applied are discussed and the conclusion is reached that the action of growth hormone is antagonistic to that of cortisone.

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Metabolism of Lactic Acid and Glycerol in C^{14}-Glyceryl Lactopalmitate.* (23786)

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The metabolism of acylated glycerol to carbon dioxide and water, in the light of current knowledge depends upon the intermediate formation of free or deacylated glycerol. Similarly, metabolism of fatty acids esterified with glycerol is dependent upon hydrolysis. The hydrolysis rates of glyceryl esters thus become a variable, along with absorption rates and possible transesterifications, determining the catabolism of glycerol. Karnovsky and Gidez(1,2) compared the appearance of CO_2 from the metabolism, in rats, of C^{14}-glycerol esterified with various acids. Esterification with oleic acid decreased the rate at

* A preliminary report was presented at meeting of Am. Soc. Pharm. and Exp. Therap., Chicago, Ill., Apr. 16, 1957. C^{14}-compounds were obtained under U. S. Atomic Energy Commission License 45481.

which carbon dioxide was eliminated from the labelled glycerol. No other essential difference in metabolism was reported. The rapidity with which carbon dioxide is eliminated from ingested glycerol(1,2) affords then, a convenient method for determining whether or not synthetic or natural glycerides are effectively utilized for energy yielding reactions. When glycerol is esterified by fusion with equimolar quantities of lactic acid and palmitic acid an ester preparation with emulsifying properties is formed. The preparation, glyceryl lactopalmitate, would be expected to contain glycerol-lactic acid ester linkages, which have never been identified as naturally occurring, although there is some evidence that pancreatic enzymes can form esters from lactic acid(3). In consequence, a study of glyceryl lactopalmitate has been conducted *in vitro*, and *in vivo* with the aid of glycerol-1,3-C^{14} and lactic acid-2-C^{14}.

Materials and methods. Glyceryl lacto-2-C^{14}-palmitate was prepared by fusion of glycerol (1M) and lactic acid (1M) and palmitic acid (1M). Sodium lactate (specific activity 0.82 mc/mM, Isotope Specialties, Inc.); was equilibrated with 80 percent lactic acid prior to esterification to introduce the carbon label. The resulting ester preparation had a saponification number 269, acid number 1.8, and a radioactivity 3.86 x 10^6 disintegrations per minute per g.† Glyceryl-1,3-C^{14}-lactopalmitate was prepared from glycerol-1,3-C^{14} (Research Specialties Company) after equilibration with 95 percent glycerol prior to formation of the ester (saponification number 255, acid number 8.1, 4.17 x 10^{10} disintegrations per minute per g). The radioactive esters dissolved in warm olive oil were administered by intubation to mongrel dogs and albino (Wistar strain) rats. Expired carbon dioxide was collected in sodium hydroxide scrubbers from rats which were maintained in glass metabolism cages. Lymph and blood samples were assayed for lactic acid by method of Barker and Summerson(4). Radioactive lactate was oxidized to acetaldehyde by a modification of the method of Lucus(5) and then collected as the dimedon derivative(6) which is conveniently counted. Samples of blood or lymph were analyzed before and after incubation (as noted in the tables). The acetaldehyde from oxidation of lactate (and that present before oxidation) was obtained as follows: One hundred ml of aliquot from the deproteinization of whole blood or lymph (1 ml of blood diluted to 10 ml) was placed in a 500 ml Kjeldahl flask with cold finger reflux condenser and addition tube drawn to a capillary tip. To this was added 10 ml of solution containing carrier (100 mg lithium lactate) and 5 ml of catalyst (0.05M iodine monochloride). The solution was brought to a boil, and 20 ml of N/5 ceric sulfate in N/2 sulfuric acid was added dropwise over a 15-20 minute period. Air was slowly aspirated through the flask during the period, and the entrained acetaldehyde was collected in 2 absorption towers containing 100 ml each of 0.4% dimedon adjusted to pH 6.0(6). After a total absorption time of ½ hour, the solutions in the towers were combined and then acidified to pH 3.9 with acetic acid. The precipitated dimedon addition product was removed by filtration, washed with water, dried at 80°C and weighed. Radioactivity was then determined. Confirmation of the radioactivity of isolated samples was achieved by recrystallization (m.p. 137-139°C) and conversion to 2,2,7,7,9-pentamethyl-4,5-dioxo-octahydroxanthene. Fecal radioactivity was determined on oven dried (99°C) samples. Urinary radioactivity was determined on dried samples made alkaline with an excess of sodium hydroxide before evaporation. Nonenzymatic hydrolysis of glyceryl monolactate (acetyl value 574, acid number 10.7, saponification number 350) was determined by dissolving 0.1263 g of ester in 300 ml of water at pH 7.3. The pH of the solution was maintained automatically at 7.3 by additions of 0.04593 N sodium hydroxide from a Beckman automatic titrator in an atmosphere of nitrogen. Concentrations of ester were calculated from alkali consumption during the reaction, at 30°C. Enzymatic hydrolysis of glyceryl lactopalmitate was determined by shaking a mixture containing 0.1211 g glyceryl lactopalmitate, 0.05 g lipase (Steapsin, Nutritional Biochemicals Corp.), 10

† Counts were obtained on infinitely thick samples using a Libby anticoincidence counter, *cf.* Libby, W. F., *American Scientist,* 1946, v44, 98.

TABLE I. Distribution of Lactate-2-C^{14} following Administration of Glyceryl Lacto-2-C^{14}-Palmitate* to 20 kg Male Dog.

Time, hr	Thoracic vol lymph, ml	Duct lymph, % admin. lactate in lymph collected
0 – ½	21	.041
½–1¼	38	.10
1¼–4	60	.034
	Blood (after 4½ hr incubation), % admin. lactate in total body blood†	
2¾	2.43	
4	1.31	
24	.10	

* 10 g glyceryl lactopalmitate in 30 g olive oil by stomach tube.

† Calculated from relation Blood vol = .78 × body wt.

ml hexane. 35 ml 2M sodium acetate in 125 ml pyrex g.s. bottle at 29-30°C. At the end of the incubation period, 125 ml of neutral 95% ethanol was added to the reaction mixture which was then titrated to the phenophthalein end point with 0.05 N sodium hydroxide. The percentage hydrolysis was calculated from the alkali consumption less control titrations of mixtures incubated without substrate.

Results. In preliminary studies with dogs, attempt was made to correlate the appearance of lactic acid and its esters in the lymph from the thoracic duct‡ with the intestinal absorption of the administered unlabelled glyceryl lactopalmitate. Polyethylene or siliconized glass cannulas were employed in these experiments. Minor adjustments in the position of the cannula, such as may be influenced by the postural habits of the dog, led to considerable variations in flow rate. Thus, effluent samples may represent lymph that is considerably changed in its chemical composition due probably to some stasis in the duct or cannula. In a duplicate determination, a heparinized sample of lymph from the thoracic duct (initial lactic acid content 101-102 mg/100 ml) after incubation at 37.5° for four and one-half hours increased its lactic acid content to 315-316 mg/100 ml; these results indicated that a variety of artifacts would make interpretation of lactic acid content of lymph difficult unless isotopically labelled material were employed.

A male dog (2.0 kg) was given 10 g of glyceryl lacto-2-C^{14}-palmitate in 30 g of olive oil by stomach tube. Samples of blood and lymph were collected and analyzed for radioactive lactate. The results of these determinations (Table I) indicate that some digestion and absorption of glyceryl lactopalmitate (presence of radioactive lactate in lymph) had taken place within one-half hour after administration of the ester. Since the radioactivity levels in both blood and lymph rose during the first one-two hour period and then fell, metabolism of the ester was indicated. Confirmation of this point was then sought on rats where collection of expired air is easily accomplished.

Following administration of glyceryl-lacto-2-C^{14}-palmitate to rats, elimination of radioactive carbon dioxide was detected for periods as long as forty-eight hours (Table II). The dose of fat employed seems to have been well-tolerated in view of the small (1.4%) amount of fecal loss and absence of diarrhea.

C^{14}, after administration of glyceryl-1-3-C^{14}-lactopalmitate to rats, was also eliminated predominantly in the form of respiratory carbon dioxide. This, together with the similar elimination from glycerol administered in the unesterified form, serves to confirm previous data(1,2). A definite conclusion on comparative rates of oxidation of free and esterified glycerol would require studies on a larger series of animals. Fecal losses are of the same order of magnitude (Table III) irrespective of the type of radioactivity administered. Since the percent of radioactivity recovered in the feces is essentially the same in animals receiving radioactive glycerol as in the animals receiving equimolar doses of glycerol-1,3-C^{14}-lactopalmitate, good absorption of the synthetic fat is again indicated.

Since both glycerol and lactic acid were metabolized following administration of glyceryl lactopalmitate it is presumed that hydrolysis of the ester linkages from both acids has taken place. Hydrolysis of glyceryl lactopalmitate was carried out *in vitro* with commercial samples of calf lipase. In a 4-hour period hydrolysis had taken place to the extent of 32%. In 24 hours, seventy-five per-

‡ The authors thank Dr. L. E. Edwards for his kind assistance.

TABLE II. Metabolism of Glyceryl Lacto-2-C^{14}-Palmitate in Female Rats.

		Percent of dose eliminated in expired air							
		Between hr intervals					Final	Final	
Wt, g	Dose,*ml	0–6	9	12	21	48	urine	feces	Total
240	3		12.2	7.2					19.4
240	3		21.4				16.7	1.4	39.5
250	3		20.4						20.4
		5 hr							
263	3	11.2							11.2
275	3.3		18.9						18.9
				13 hr					
290	3.4			24.6			7.9		32.5
300	3.6				41.6	9.2	3.9	1.4	56.1
390	4.7				51.3	4.7	4.4		60.4

* 10 g of glyceryl lacto-2-C^{14}-palmitate dissolved in 30 ± g of olive oil. 1 ml of solution assayed 2.01×10^6 dpm.

TABLE III. Metabolism of Glyceryl-1,3-C^{14}-Lactopalmitate in Male Rats.

		Percent of dose eliminated in expired air							
		Between hr intervals					Final	Final	
Wt, g	Compound	0–6	9	21	31½	48	urine	feces	Total
						51½ hr			
190	Glyceryl-1,3-C^{14}-lactopalmitate*		46.4	19.6	7.5	4.2	6.2	1.4	85.3
180	*Idem*	27.5		31.6		9.2	3.8	.91	73.0
243	.88 ml olive oil, 50 mg glycerol	43.1		35.3		4.6	4.3	1.4	88.7
213	*Idem*†	43.8		27.0		4.5	3.6	1.7	80.6

* Radioactive ester (10 g) was dissolved in 30 g olive oil, as in previous series. One ml of solution contained by assay 7.77×10^6 dpm.

† 3.83×10^7 dpm glycerol. Total free glycerol present equals amount of glycerol present as ester in the dose of glyceryl lactopalmitate.

cent hydrolysis was achieved. This latter hydrolysis demonstrates the cleavage of both palmitic and lactic acid ester linkages and confirms the interpretation made from the tracer studies. In the absence of enzyme no significant hydrolysis was observed. Hydrolysis of glyceryl lactate proceeds to a significant degree at pH 7.3 in the absence of enzyme. In the titration study, 50% of the product underwent hydrolysis in 550 minutes. Further experimentation would be required to determine whether or not the hydrolysis of glyceryl lactate ester linkages is enzymatically controlled *in vivo*.

Summary. 1. The fate of lactic acid-2-C^{14} following administration of glyceryl lacto-2-C^{14}-palmitate was studied by converting lactate from blood or lymph to acetaldehyde which was counted as the dimedon derivative and as 2,2,7,7,9-pentamethyl-4,5-dioxo-octahydroxanthene. 2. The lactic acid content of lymph samples from the thoracic duct of the dog is subject to variation which arises from the speed with which samples are obtained through a cannula. 3. Appearance of radioactive CO_2 in the expired air of rats following the administration of glyceryl lacto-2-C^{14}-palmitate or glyceryl-1,3-C^{14}-lactopalmitate, as well as the low fecal radioactivities, indicates a high degree of utilization of the fat. 4. In comparison with glycerol and glyceryl esters the metabolism of glyceryl-1-3-C^{14}-lactopalmitate, as determined by the radioactivity of expired CO_2, follows a path consistent with that expected from a normal ester of glycerol.

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Increase in Circulating Red Cell Volume of Normal Rats after Treatment with Hydrocortisone or Corticosterone. (23787)

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The humoral regulation of red cell production has been the subject of several recent investigations(1,2,3,4,5). However, there is very little direct evidence that the adrenocortical hormones are capable of exerting a significant influence on the hematocrit, red cell count, hemoglobin or red cell volume.

The present studies were undertaken to determine the effects of prolonged daily injections of hydrocortisone and corticosterone on the blood of the normal rat. This was deemed of fundamental interest because of previous reports showing that injections of a purified ACTH preparation in normal mice(6) and rats(7) caused an elevation in hematocrit and total red cell volume.

Materials and methods. Male albino rats of the Wistar (Purdue) strain were studied in 6 groups: Group (1) 6 normal controls, (2) 8 rats given hydrocortisone at a dose of 0.5 mg/kg/day, (3) 10 rats at a dose of 2 mg hydrocortisone/kg, (4) 8 rats at 5.0 mg hydrocortisone/kg, (5) 10 rats at 5 mg corticosterone/kg, and (6) 8 rats at 10 mg corticosterone/kg. The Hydrocortisone Acetate† or Corticosterone† was suspended in 0.9% NaCl solution and injected subcutaneously daily (6 days/week) at various sites under the skin of the back. Blood was obtained for erythrocyte, hemoglobin and hematocrit determinations by anesthetizing the rat with ether and clipping the tail. Erythrocyte counts were done with U.S. certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Van Allen hematocrit tubes (1.6% aqueous sodium oxalate as diluent), spun 30 minutes at 3000 rpm with radius of 17 cm. Hemoglobin determinations were made on Klett-Summerson colorimeter by the acid hematin method(8). Rats were weighed at the time of hematologic studies. Erythrocyte blood volume was determined with P^{32} tagged erythrocytes using the method of Hevesy(9) as modified by Berlin(10). Each animal was anesthetized with ether and the tagged cells were injected into a vein, exposed by incision on inner aspect of thigh and allowed to mix for 10 minutes, then a blood sample was removed *via* heart puncture for counting. P values were determined by using Fisher's "t" test for statistical significance.

Results. The data are presented in Table I. Hematocrit, erythrocyte, hemoglobin and total red cell volume values at 60 days were significantly elevated for all dosages of hydrocortisone and corticosterone. P values of less than .01 were obtained for all 60 days values except the hematocrit for the 0.5 mg dose of hydrocortisone which was 0.1 and the hemoglobin and hematocrit for the 5 mg dose of hydrocortisone which were 0.02 and 0.07 respectively. There was a decrease in mean corpuscular volume upon treatment with either hydrocortisone or corticosterone. Therefore, hematocrits and total red cell volumes were not elevated to the same degree as red cell counts.

The most striking results were obtained with daily injections of hydrocortisone at 2 mg/kg. After 60 days of treatment, the erythrocyte count had increased from 9.5 to 13.9 million cells/cu mm; the hematocrit, from 43.5 to 52.1%; the hemoglobin, from 16.2 to 18.0 g/100 cc; and red cell volume was elevated from 1.96 to 3.2 cc/100 g body weight. The mean corpuscular hemoglobin decreased along with mean corpuscular volume. The mean corpuscular hemoglobin concentration remained essentially normal throughout treatment. Ninety day erythrocyte counts, hematocrit and red cell volumes for the 2 mg hydrocortisone dosage were

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† Hydrocortisone Acetate and Corticosterone (U-4460) were supplied through the courtesy of Upjohn Co., Kalamazoo Mich.

TABLE I. Effects of Hydrocortisone and Corticosterone on Blood Picture of Normal Adult Male Rats.

Day	No. rats	Body wt, g	RBC, millions/mm³	Hemato-crit, %	Hemoglobin, g/100 cc	M.C.V., μ^3	M.C.H., $\mu\mu g$	M.C.H. conc., %	
				Normal controls—no treatment					
0	6	251.8 ± 35	8.8 ± .45	44.0 ± 1.9	15.9 ± .21	50.0 ± 2.7	18.1 ± .88	36.2 ± 1.5	
10	6	277.8 ± 20	9.7 ± .51	44.6 ± 1.9	16.4 ± .40	45.9 ± 2.9	16.9 ± .78	36.8 ± 2.0	
34	6	330.8 ± 11	9.2 ± .77	45.5 ± 4.0	16.6 ± 2.0	49.6 ± 6.7	18.1 ± 2.9	36.5 ± 1.9	
40	6	340.2 ± 13	9.5 ± 1.47	46.3 ± 3.7	16.2 ± 1.0	49.3 ± 6.2	17.3 ± 2.3	35.1 ± 1.8	
50	6	347.7 ± 17	9.3 ± 1.25	44.0 ± 1.8	15.8 ± .84	48.1 ± 7.8	17.3 ± 3.3	35.9 ± 2.2	
60	6	373.0 ± 17	9.7 ± .79	45.2 ± 2.3	16.0 ± .55	46.7 ± 2.7	16.7 ± 1.3	35.5 ± 1.8	
76	5	390.6 ± 28	9.6 ± .79	44.8 ± 1.5	16.1 ± .75	47.0 ± 5.6	16.8 ± 1.8	35.9 ± 1.6	*
90	5	398.2 ± 21	8.5 ± 1.3	44.8 ± 3.3	15.7 ± .46	53.7 ±11.2	18.8 ± 3.2	35.2 ± 2.2	†
				Corticosterone—5 mg/kg/day					
0	10	319.0 ± 19	9.7 ± .92	44.4 ± 2.1	16.0 ± .70	46.1 ± 4.6	16.7 ± 2.0	35.9 ± 1.4	
10	9	316.6 ± 23	10.8 ± 1.7	44.0 ± 3.3	16.5 ± 1.8	41.3 ± 5.2	15.5 ± 2.6	37.5 ± 2.3	
20	8	333.1 ± 24	11.9 ± 1.0	46.3 ± 2.6	16.5 ± 1.1	39.0 ± 3.2	13.9 ± 1.2	35.8 ± .84	
40	8	360.4 ± 35	10.4 ± .36	42.3 ± 1.0	16.9 ± .64	40.6 ± 1.5	16.3 ± .96	40.1 ± 2.1	
60	8	376.4 ± 37	12.3 ± 1.2	49.0 ± 2.6	17.5 ± .53	39.8 ± 6.0	14.2 ± 1.4	35.8 ± 1.3	‡
				Corticosterone—10 mg/kg/day					
0	8	360.8 ± 38	9.1 ± .83	42.3 ± 2.3	15.7 ± .79	46.6 ± 4.6	17.3 ± 2.2	37.2 ± 1.7	
10	8	337.6 ± 56	13.1 ± .90	46.6 ± 3.0	17.9 ± 2.3	35.6 ± 3.3	13.7 ± 1.1	38.5 ± 2.1	
20	7	334.4 ± 49	11.8 ± .77	45.3 ± 1.7	16.8 ± .64	38.5 ± 3.5	14.2 ± 1.1	37.1 ± 2.0	
60	6	361.8 ± 55	12.0 ± 1.2	50.5 ± 2.2	18.4 ± 1.1	42.3 ± 4.9	15.4 ± 1.7	36.4 ± 1.9	§
				Hydrocortisone—.5 mg/kg/day					
0	8	302.3 ± 43	10.1 ± .60	44.1 ± 3.6	16.8 ± 1.3	42.3 ± 6.9	16.6 ± 1.4	38.2 ± 1.5	
20	8	315.1 ± 32	9.8 ± 2.20	45.4 ± 1.6	17.6 ± .71	48.0 ±11.6	18.8 ± 4.5	38.7 ± 1.6	
34	7	328.3 ± 44	10.7 ± .59	46.1 ± 2.7	16.4 ± .89	43.2 ± 1.7	15.4 ± 1.1	35.6 ± 1.8	
40	7	333.9 ± 45	11.2 ± .66	46.0 ± 2.8	17.3 ± .99	40.9 ± 2.8	15.3 ± 1.4	37.7 ± 3.1	
60	7	355.1 ± 52	12.6 ± .66	46.6 ± 1.3	18.0 ± 1.3	37.1 ± 2.2	14.3 ± 1.2	38.7 ± 1.8	
90	7	370.9 ± 56	10.8 ± 1.5	48.3 ± 3.3	17.4 ± .87	45.4 ± 7.1	16.3 ± 2.8	36.1 ± 2.4	‖
				Hydrocortisone—2 mg/kg/day					
0	10	272.1 ± 24	9.5 ± .98	43.5 ± 2.2	16.2 ± 1.0	46.4 ± 5.6	17.1 ± 2.0	37.2 ± 1.0	
10	6	262.2 ± 27	10.5 ± 1.9	47.5 ± 4.4	17.3 ± 1.3	45.9 ± 6.8	16.9 ± 2.5	36.6 ± 1.2	
34	9	304.8 ± 29	11.5 ± 1.3	50.2 ± 1.7	18.3 ± 1.0	44.2 ± 5.6	16.1 ± 2.7	36.4 ± 1.8	
40	9	301.6 ± 28	12.3 ± .99	49.4 ± 1.6	17.6 ± .86	40.5 ± 3.0	14.4 ± 1.4	35.6 ± 2.0	
60	9	315.4 ± 28	13.9 ± 2.0	52.1 ± 1.5	18.0 ± 1.3	38.8 ± 8.3	13.3 ± 2.5	34.6 ± 1.8	¶
90	7	326.1 ± 11	12.1 ± 1.5	50.4 ± 1.1	18.6 ± .50	42.3 ± 5.4	15.6 ± 2.1	37.0 ± 1.6	**
				Hydrocortisone—5 mg/kg/day					
0	8	300.3 ± 26	9.4 ± .72	44.5 ± 2.7	15.8 ± .84	47.5 ± 3.4	16.9 ± 1.4	35.6 ± 1.1	
10	8	293.9 ± 36	11.8 ± 1.2	48.1 ± 4.0	18.7 ± 1.5	41.3 ± 6.8	16.0 ± 2.3	39.0 ± 2.2	
20	8	279.9 ± 35	12.6 ± 1.4	48.6 ± 3.9	18.1 ± .99	38.8 ± 3.4	14.4 ± 1.2	37.4 ± 2.0	
40	7	307.3 ± 37	12.1 ± .80		18.7 ± .92		15.4 ± 1.1		
60	7	300.0 ± 41	12.0 ± 1.4	49.7 ± 5.7	18.3 ± 1.2	38.8 ± 4.9	15.4 ± 1.4	37.1 ± 2.5	††

Day = Day of treatment; M.C.V. = Mean corpuscular vol; M.C.H. = Mean corpuscular hemoglobin.

Red cell vol (cc red cells/100 g body wt) are as follows:

* 1.96 ± .07 ‡ 2.85 ± .31 ‖ 2.73 ± .30 ** 2.80 ± .52

† 2.28 ± .08 § 2.90 ± .13 ¶ 3.21 ± .46 †† 2.90 ± .28

± = stand. dev.

lower than 60 day values but not to a significant degree (P values for red cell volume, hematocrit and erythrocyte counts were .40, .10 and .08 respectively).

Treatment with hydrocortisone at a dosage of 5 mg/kg daily resulted in a less pronounced rise in blood values than the 2 mg/kg dose, and may be the result of toxicity indicated by failure of rats in this group to gain weight. The 0.5 mg dose of hydrocortisone induced a slower and more gradual rise in blood values than either the 2 mg or 5 mg dose.

Rats treated with corticosterone at daily dosage of 5 and 10 mg/kg for 60 days showed a significant elevation in hematocrit, hemoglobin, red cell count and total red cell volume but to a lesser degree than those treated with hydrocortisone at 2 mg/kg. Sixty days of treatment with corticosterone at a daily dose of 10 mg/kg increased erythrocytes from 9.1

to 12.0 million cells/cu mm; the hematocrit, from 42.3 to 50.5%; hemoglobin, from 15.7 to 18.4 g/100 cc; and red cell volume to 2.9 cc/100 g body weight (1.48 times untreated controls). As with rats receiving hydrocortisone at a dosage of 2 mg/kg, these rats also showed a decreased mean corpuscular cell volume and mean corpuscular hemoglobin. The mean corpuscular hemoglobin concentration remained relatively normal.

A group of 10 rats not included in Table I was treated with 10 mg hydrocortisone/kg daily via subcutaneous injection. Progressive loss in weight and ulcerations at injection sites were noted. Only 2 animals survived in this group at 50 days and none at 60 days. Increases in erythrocytes, hematocrit and hemoglobin were noted at 10 days but these values gradually decreased to below-normal values as toxic signs appeared.

Discussion. Hydrocortisone and corticosterone produced elevation in erythrocytes, hematocrit, hemoglobin and total red cell volume (Table I) in many respects similar to that produced by ACTH(7), hypoxia(11) and cobalt(12). Two mg hydrocortisone/kg for 60 days (Table I) increased the hematocrit to approximately the same degree as rats treated for 40 days with 0.5 mg cobalt nitrate/day subcutaneously(12) or 1 mg ACTH intraperitoneally for 116 days(7). Hydrocortisone produced a more marked effect than corticosterone judged both by rate and magnitude of response shown in Table I.

Several factors in addition to the present results suggest that adrenocortical secretions play a significant role in regulation of hematopoiesis. Removal of the adrenal gland in the rat results in a temporary anemia(13,14), corrected upon development of accessory adrenals(13). Administration of adrenocortical extract in rats(15) or cortisone in rats and dogs(15,16) repaired the anemia of adrenalectomy. Cushing's disease is accompanied by a polycythemia(17). An increase in "stem" and erythroid cells was noted in bone marrow from rats treated for a short time with corticosterone(2). Adrenalectomized rats did not show the characteristic polycythemic response to cobalt injections (13).

Consideration should be given to the possibility of dissociation of total body growth and growth of red cell mass. However, we have found that animals treated with 2 mg hydrocortisone/kg for 60 days have a significant elevation in red cell volume for animals of their age, and weight. Therefore, elevation in total red cell volume cannot be due to a repression of normal weight gain.

Recent work has shown that adrenalectomized rats respond to bleeding with an increase in plasma erythropoietin, but the response is less than that shown by normal rats (18). The demonstration by Gley(5) of the existence of more than one erythropoietin in plasma could explain the response of adrenalectomized animals to bleeding.

The increased red cell volume after treatment with the adrenocortical hormones (Table I) is interesting when considered along with the greatly increased adrenal weights and erythropoietic stimulation in rats maintained at depressed oxygen(19) or treated with cobalt(13). The mechanism by which bone marrow is stimulated by adrenocortical hormones is not clear. The work of Gross(20) with bone marrow *in vitro* would indicate that the adrenocortical hormones stimulate bone marrow directly.

Conclusions. 1. Administration of hydrocortisone or corticosterone for 60 days to normal rats elevated the total circulating red cell volume from 1.45 to 1.64 times that of normal untreated controls. 2. Rats treated with 0.5, 2 or 5 mg hydrocortisone or 5 or 10 mg corticosterone/kg body weight for 60 days, showed a significant elevation in erythrocyte, hematocrit and hemoglobin values.

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Hemolytic Anemia in Rabbits Following Injection of Bacterial Endotoxin. (23788)

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Alterations in the numbers of circulating white cells and platelets after injections of Gram negative bacterial endotoxins are well known(1,2). Corresponding changes in the red cells have not been as clearly defined. Braude *et al.* found variable effects of *E. coli* endotoxin on the blood volume and hematocrit of rabbits, with little change in the circulating red cell mass, although leukopenia and neutropenia occurred(3). Similarly, Kopp noted little change in the hemoglobin levels of patients receiving typhoid-paratyphoid (TAB) vaccine for production of therapeutic fever(4). Cartwright *et al.*(5) found that injecting 5 doses of typhoid vaccine intramuscularly into dogs did not induce the hypoferremia and anemia that occurs during infection. Willison observed that various bacterial exotoxins depressed the reticulocyte response to a constant blood loss anemia in rabbits but the effect of bacterial endotoxins was not investigated(6). It will be shown here that rabbits may develop a mild hemolytic anemia after a single injection of endotoxin and that they invariably develop such anemia after multiple injections of endotoxin derived from typhoid bacilli.

Materials and methods. Male albino buck rabbits, weighing 5-6 lb at the beginning of the experiments, were used. *Endotoxin.* Trypsin treated *S. typhi* (Ty2) endotoxin was prepared as previously described(7). Briefly, an overnight culture† was killed with chloroform, digested with trypsin, precipitated with acetone and extracted by repeated alcohol-ether precipitations from aqueous solution. The final solution, in physiological saline, was heated at 56° for 30 minutes in a sterile vial and stored at –25°C. The LD_{50} of this endotoxin injected intravenously into 5-6 lb rabbits varied between 0.01-0.1 ml of an undiluted solution containing 10 mg per ml of endotoxin by dry weight. *Hematological studies.* Whole blood and plasma hemoglobin concentrations, reticulocyte counts, and mechanical and osmotic fragility of red cells were determined by the usual methods(8). The hemoglobin levels of apparently normal rabbits were found to vary from 10.5 to 14 g %, and the reticulocyte counts from 0.6 to 3.0%. *Labelled red cells.* Five ml of blood was drawn by cardiac puncture and the red cells labelled with 40-70 microcuries of radioactive sodium chromate ($Na_2Cr^{51}O_4$) according

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to the method of Jandl *et al.*(9). Aliquots of blood (0.02-0.1 ml) were hemolyzed in 3 ml of distilled water and the radioactivity determined in a well-type scintillation counter. The blood specimen obtained 24 hours after injection of the labelled cells was considered to have 100% radioactivity and subsequent values were plotted semilogarithmically without correcting for elution of Cr^{51}(10). The half survival time (T ½) of the labelled red cells was determined graphically, and was found to average 14.5 days in normal rabbits. The value obtained by Donahue *et al.* was 12 days(10). The "PVP" (polyvinyl pyrrolidone) test was used to detect sensitization of red cells; 5% PVP (K-44) solution buffered at pH 7.4 was used(11). The Coombs test was performed on saline washed red cells using antisera obtained from rats that had received multiple injections of rabbit serum.

Results. Effect of a single intravenous injection of endotoxin. Each of 6 rabbits was given a single dose of endotoxin intravenously; 0.1 ml undiluted was given to 2 and 0.1 ml of a 1:8 dilution to the other 4. Varying degrees of mild reticulocytosis not exceeding 5% with corresponding decreases in the hemoglobin concentrations of 1-2 g % were observed from the 4th to the 15th day after the injections of endotoxin in one of the rabbits that received undiluted and in 2 of those that received diluted endotoxin. In the other 3 rabbits, no such changes were noted. Red cell survival time was studied before and after injection of endotoxin in the 2 rabbits receiving 0.1 ml of undiluted endotoxin. The T ½ was normal for both rabbits (15.5 and 14.5 days respectively) prior to injection of endotoxin. However, 9 days after the endotoxin had been given the red cells of one rabbit disappeared at a rate corresponding to a T ½ of 9.5 days. The decreased survival of the red cells occurred during the period when the reticulocytosis and lowered hemoglobin levels were observed. No changes in the survival time of the red cells were observed in the other rabbit, and there were no significant changes in hemoglobin and reticulocyte levels.

Effect of multiple injections of endotoxin. Three intravenous injections of 0.05 ml of a 1:8 dilution of endotoxin were given during 5 days to each of 5 rabbits. Two of the 5 rabbits developed minimal reticulocytosis 12 days after the first injection. In another experiment, 5 rabbits each received a total of 9 injections of 0.05 ml of a 1:8 dilution of endotoxin over a period of 2 weeks; 4 of these 5 developed mild reticulocytosis between the 10th and 20th days after the first injection and there were small corresponding decreases in the hemoglobin levels. Finally, large doses of endotoxin were given daily for about 3 weeks to 4 "immune" rabbits which had received multiple doses of endotoxin in the past, but had not received endotoxin during the preceding month or two. The reticulocyte and hemoglobin levels in such animals are invariably normal after one month's rest from injections. Two of these "immune" animals received multiple injections of 0.5-0.1 ml of a 1:4 dilution of endotoxin and the other two received daily 0.05-0.30 ml of undiluted endotoxin in increasing doses. The observations on one rabbit of each group are shown in Fig. 1 and 2. In all 4 animals, reticulocytosis first appeared 7-10 days after injections of endotoxin were begun and maximal reticulocyte counts of 8-12% occurred subsequently. Hemoglobin concentrations were decreased in all the animals; the maximal change being a reduction from 12 to 8 g %. That increased destruction of red cells occurred as a result of repeated injections of endotoxin is shown in Fig. 2. The red cell survival studies in this rabbit showed that the T ½ fell to 7 days, and indicated that the accelerated destruction

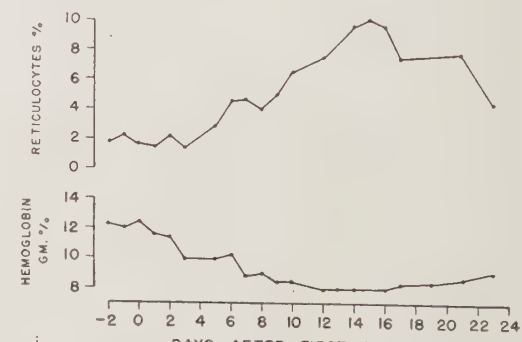

FIG. 1. Effect of multiple injections of endotoxin on reticulocyte counts and hemoglobin concentrations in blood of a rabbit.

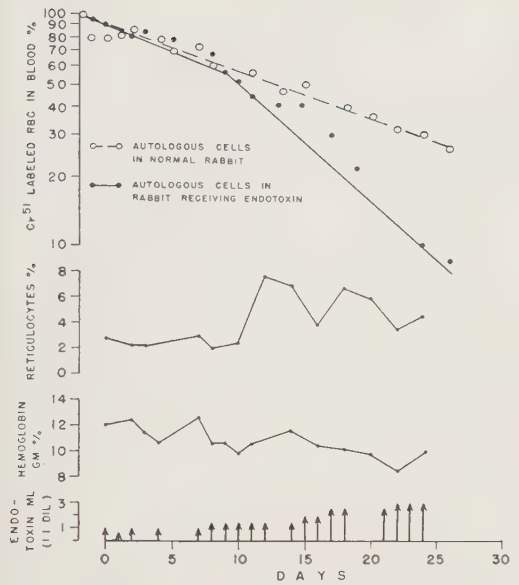

FIG. 2. Effect of multiple injections of endotoxin on red cell survival, reticulocyte counts and hemoglobin concentrations in blood of a rabbit.

of the red cells began about 8 days after the first injection of endotoxin. In all 4 "immune" animals the hemolytic process was self-limiting; despite continued injections of endotoxin the reticulocyte counts decreased and hemoglobin levels increased during the third or fourth week after the first injection of endotoxin.

Investigations of mechanisms of hemolysis following injection of endotoxin. 1. *In vitro* agglutinating effects of endotoxin were investigated by adding a solution containing 10 mg/ml of endotoxin to an equal volume of a 10% suspension of washed or unwashed red cells in autologous plasma from normal animals. No gross or microscopic agglutination and no hemolysis were observed after one-half hour's incubation at 37°C. Similarly, addition of endotoxin to washed or unwashed red cells in autologous serum from hyperimmune animals did not cause agglutination. Agglutination of red cells in the presence of endotoxin could be produced only if the red cells were allowed to incubate with endotoxin before antibody was added.

2. In order to determine whether administration of endotoxin to a hyperimmune animal would induce hemolysis *in vivo*, a hyperimmune rabbit was given 1.0 mg of endotoxin intravenously. Within the next hour the hemoglobin concentration of the blood increased by 2.3 g % and returned to pre-treatment values during the next 24 hours. There was neither microscopic autoagglutination of the red cells nor evidence of sensitization of the red cells, as determined by the PVP test. A second hyperimmune rabbit was given 100 mg of endotoxin intracardially. As in the previous animal, the hemoglobin concentration of the blood rose transiently by 1.2 g % over pre-treatment values during the hour after injection, and the plasma hemoglobin rose from 18.9 mg % to 56.7 mg % during the 15 minutes after the injection of this large dose of endotoxin. The animal died 2½ hours after the injection.

3. When red cells were obtained at various times during the course of the hemolytic response to the injection of endotoxin into immune rabbits, these cells showed no abnormality of osmotic or mechanical fragility. Such red cells did not agglutinate in the presence either of PVP or of antisera to rabbit serum (Coombs' test), indicating that these cells were probably not sensitized with autologous serum proteins.

4. Since the hemolytic response occurred a week or more after the injection of endotoxin and was more profound in animals that had received multiple doses of endotoxin, the effect of passive transfer of antibody to endotoxin was studied. Each of 2 rabbits was given 10 ml of serum from a rabbit that had received multiple injections of endotoxin. The antiserum agglutinated endotoxin-coated red cells to a titer of 1:8192(12). A third rabbit received 10 ml of serum from an untreated donor. Each animal was then given 0.05 ml of a 1:8 dilution of endotoxin. Only one of the animals that received immune serum showed a transient reticulocytosis to 4.5%, and this occurred 10 days after the injection of endotoxin. Such a response is consistent with that of normal animals receiving a single dose of endotoxin. The passive transfer of antibodies to endotoxin did not appear to augment or accelerate the hemolytic process that occurs after an injection of endotoxin.

5. A rabbit was made anemic by giving re-

peated doses of endotoxin. The red cells of the anemic rabbit, when transfused into a normal recipient rabbit, had a normal survival time (T ½ = 14 days).

6. Splenomegaly was found uniformly in the animals that had received multiple doses of endotoxin. Uninjected rabbits of the size used in these experiments have a mean splenic weight of about 0.4 g per kg body weight, whereas the animals that received repeated doses of endotoxin had a mean splenic weight of 1.6 g per kg body weight. The role of the spleen in the pathogenesis of the hemolytic anemia herein described was investigated by giving a non-immune, splenectomized rabbit 3 injections of endotoxin. The animal developed transient reticulocytosis and a decreased hemoglobin concentration in the blood beginning on the 12th day after injection of endotoxin. Thus, splenectomy did not abolish the hemolytic response to the injection of endotoxin.

Discussion. Hemolytic anemias may be accompanied by abnormalities in the red cells or in the host. The hemolytic anemia that follows the injection of endotoxin is probably not related to an abnormality of the red cell. This is supported by the absence of sensitization *in vitro*, the normal mechanical and osmotic fragilities, and particularly the normal survival in normal recipients of cells from anemic animals. The absence of sensitization makes it unlikely that the hemolytic anemia is due to attachment *in vivo* of endotoxin to red cells, followed by agglutination and hemolysis in the presence of anti-endotoxin antibody(13). However, the possibility cannot be excluded that cells coated with endotoxin *in vivo* were removed rapidly from the circulation of immune animals and hence were not detectable peripherally. Such an occurrence might explain the hemoglobinemia observed in the immune animal shortly after injection of a large dose of endotoxin.

The role of antibody to endotoxin in the production of the hemolytic response is not clear. The hemolytic response occurs during the second week after the injection of single or multiple doses of endotoxin, suggesting that antibody may be involved. On the other hand, the presence of antibody alone is insufficient to induce hemolysis since hyperimmune animals with high titers of antibody may not be anemic, and since partial recovery from the hemolytic anemia may occur while the injections of endotoxin are still being given. Furthermore, the passive transfer of antibody to normal recipients did not accelerate or augment the reticulocyte response to the subsequent injection of a single dose of endotoxin.

It appears likely from the foregoing evidence that the mild hemolytic anemia occurring after injection of endotoxin may be secondary to an alteration in the host tissues. The latent period of a week or more that is required for development of anemia after injection of endotoxin may represent a lag period during which mechanisms that increase red cell destruction are activated. Since the anemia is more apparent after repeated doses of endotoxin, at a time when the spleen is enlarged, the analogy to experimental hemolytic anemias such as those produced by injections of methyl cellulose in rats is suggested(14). However, the anemia herein described was not well correlated with the size of the spleen. The largest spleen (2.5 g/kg body weight) was found in a hyperimmune rabbit that was no longer anemic. Furthermore, splenectomy did not appear to eliminate the hemolytic response to endotoxin.

Since injection of endotoxin is followed by functional changes in the reticulo-endothelial system(2) it is conceivable that unusual activation of this system accounts for the hemolytic anemia. Repeated injection of endotoxin in rabbits produces increased granulocytic hemopoiesis and increased numbers of phagocytes in the spleen, lymph nodes and bone marrow(15). One of the functional consequences of such stimulation may be an increased capacity for random red cell destruction.

A similar hemolytic anemia occurs in mice after injection of zymosan(16) and there are indications that reticuloendothelial stimulation may account for the observation here also.

Anemia is a frequent finding in pyelonephritis and in bacteremias of various etiologies. These anemias are occasionally hemoly-

tic(17). It is conceivable that the release of endotoxin may play a role in the pathogenesis of hemolytic anemias during infection.

Summary. 1. A mild hemolytic anemia secondary to intravenous injection of bacterial endotoxin into rabbits is described. The anemia is characterized by reticulocytosis, decreased hemoglobin levels in the blood, and decreased survival of red blood cells. 2. Single injections of endotoxin occasionally produce a mild hemolytic response in rabbits during second week after injection. Repeated injections of endotoxin lead to more striking hemolytic changes which also reach their peak during second week after first injection. The hemolytic process tends to abate despite repeated administration of endotoxin. 3. Evidence is presented to indicate that the hemolytic response is not due to demonstrable changes in the red cells of the animals receiving endotoxin. There is no change in osmotic or mechanical fragility of the red cells, no sensitization to appropriate anti-rabbit-protein antisera or to polyvinyl pyrrolidone, and no evidence of hemagglutination *in vivo* during the hemolytic episode. In addition, there is no decrease in the life span of red cells when these are taken from an anemic rabbit and transfused into a normal one. 4. Presence of antibody does not seem to play a critical role in development of hemolytic anemia, and the presence of the spleen is not essential for the hemolytic response to endotoxin. 5. It is suggested that the hemolytic response is most likely due to an as yet unexplained activation of the reticulo-endothelial system.

We are indebted to Dr. Allen Erslev for bringing to our attention the hemolytic phenomenon herein described, and to Drs. William B. Castle and James H. Jandl for helpful criticisms and suggestions. Miss Mary I. Kendrick and Miss Lorraine Martin gave valuable technical assistance.

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Effect of Certain Benzimidazoles and Related Compounds Upon Azo Dye Destruction by Liver Homogenates.* (23789)

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Benzimidazole, 2,5-dimethylbenzimidazole and 2-ethyl-5-methylbenzimidazole inhibit formation of liver tumors in rats when fed with the carcinogenic azo dye, 3′-methyl-4-dimethylaminoazobenzene(1). These benzimidazoles also inhibit incorporation of glycine into heme by chicken erythrocytes(2,3), inhibit multiplication of influenza virus(4), and certain benzimidazoles and chemically related substances inhibit firefly luminescent reaction (5). In an attempt to obtain information on the mechanism of action of the benzimidazoles on the azo dye carcinogenic process, the effect of these compounds and certain chemically related compounds has been tested on azo dye destruction by liver homogenates(6,7). Iodoacetate, *p*-aminophenol, hydroxylamine, cyanide, azide and atabrine have previously been reported to inhibit dye destruction when added to the *in vitro* system(8). Of these, atabrine has been shown to affect azo dye carcinogenesis(9). The present study indicates that several compounds chemically related to the benzimidazoles also inhibit the destruction of an azo dye by liver homogenates, and this inhibition can be overcome to some extent by flavin adenine dinucleotide.

Methods. The method of measuring dye destruction by liver homogenates was essentially that described by Mueller and Miller (6,7). Rat liver homogenates (5 to 10%) were prepared in 0.01 M phosphate buffer pH 7.4 and were added last to the ice-cold reaction mixture. The azo dye, 4-dimethylaminoazobenzene, was dissolved in ethyl alcohol, and 60 μg of the dye (0.1 ml of solution) served as the substrate. Incubation was carried out at 37°C for 30 minutes at which time the reaction was stopped by the addition of ethanol-acetone-trichloracetic acid solution, and the red color due to remaining dye was measured (after proper dilution) in the Evelyn photoelectric colorimeter with 515 mμ filter. The benzimidazole derivatives and certain of the other compounds were dissolved in the alcohol solution with the dye to give the desired concentration. This was necessary since it was found that over 0.1 ml of alcohol in the reaction flask was inhibitory. Water soluble materials were dissolved in the phosphate buffer. The final volume in all reaction flasks at incubation was 3 ml. Flasks to which no homogenate had been added served as controls. It was found that the color in these controls was the same as found if the reaction were stopped at zero time.

Results. Of the various types of compounds tested, it was found that certain of the benzimidazole, benzothiazole, indole, and quinoline derivatives were effective inhibitors of azo dye destruction *in vitro* at a level of 1 mg/3 ml in the reaction flask (Table I). The 5,6-dimethyl, 2,5-dimethyl, and the 2-ethyl-5-methylbenzimidazole were the most effective inhibitors of the benzimidazoles. All of the indole derivatives except tryptophan were active inhibitors. Auramine at a level of only 20 μg/reaction flask was very effective in inhibiting the destruction of the dye. Since 2,5-dimethyl and 2-ethyl-5-methylbenzimidazole are very effective in inhibiting tumor formation when fed in conjunction with a carcinogenic azo dye, further studies on the effect of various concentrations and upon the reversal of the inhibition were done with these compounds. At a concentration of 125 μg/flask, 2,5-dimethylbenzimidazole resulted in very little inhibition while the 2-ethyl-5-methyl derivative at this concentration still inhibited dye destruction about 20%. Only slight inhibition was noted when the concentration of the latter compound was reduced to 62 μg/flask.

Addition of 25 μg of riboflavin or 20 μg of pyridoxal phosphate did not affect rate of dye

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TABLE I. Inhibition of Azo Dye Destruction by Various Chemicals.* (1 mg of compound/3 ml of reaction mixture.)

Compound	% inhibition	Compound	% inhibition
Benzimidazoles		*Misc.*	
unsubstituted	0	quinoline	12
2-methyl[1]	15	2-methylquinoline	60
5- "	9	benzotriazole	0
5,6-dimethyl[2]	58	5-methylbenzotriazole	0
2,5- " [1]	54	benzoxazole	5
2-ethyl-5-methyl[1]	61	2-methylbenzoxazole	0
5-chloro[1]	0	2-amino-1,3,4-thiadiazole[3]	0
2-methyl-5-chloro[1]	8	2-acetamido-1,3,4-thiadiazole[3]	0
2-ethyl-5-chloro[1]	4	2-ethylamino-1,3,4-thiadiazole[3]	0
5-nitro[1]	11	dinitrophenol	0
2-methyl-5-nitro[1]	17	methylene blue	0
2-ethyl-5-nitro[1]	18	Na ascorbate	0
2-mercapto	4	cysteine	0
2-hydroxy[2]	0	cystine	0
Benzothiazoles		adenine	0
unsubstituted	15	azaguanine	0
2-methyl	16	6-mercaptopurine	0
2-amino	9	thymine	0
2-phenyl	14	toluene-3,4-diamine	0
2-chloro	80	auramine (20 μg)	62
Indole			
unsubstituted	35		
2-methyl	83		
3- " (skatole)	68		
5- "	72		
tryptophan	0		

* Inhibition varied somewhat with different homogenates. The % inhibition indicated is representative, but small differences between different chemicals may not be significant.

We wish to thank Dr. L. D. Abbott, Jr., Medical College of Virginia[1]; Dr. K. Folkers, Merck and Co.[2], and Dr. R. B. Angier, Lederle Laboratories[3] for certain of the above chemicals.

destruction whether or not an inhibitor was added. However, addition of 9 μg of flavin adenine dinucleotide (FAD), or 10 μg of riboflavin phosphate (RP), was effective in partially counteracting the inhibition. Of the above 2 benzimidazoles, this effect was more noticeable with the less active 2,5-dimethyl derivative, and the effect was more pronounced at lower levels of the inhibitors (Fig. 1). Increased amounts of either riboflavin cofactor had no additional effect. A similar effect of FAD and RP was noted with the other compounds which were active inhibitors.

Discussion. It has been postulated that at least part of the protective effect of dietary riboflavin against carcinogenic azo dyes may be due to its participation in reductive cleavage of the dye forming non-carcinogenic amines(7). The present experiments add additional evidence that FAD is involved in the destruction of dye by liver homogenates. However, contrary to the finding of Mueller and Miller with their system(7), we did find that riboflavin phosphate also was somewhat effective. Our results would indicate that on a molar basis, FAD was approximately twice as active as the mononucleotide.

The rate of dye destruction by liver homogenates or slices can be altered by dietary level of riboflavin(10), methylcholanthrene (11), or iodinated casein(12). These also have an effect upon the incidence of liver tumors resulting from feeding an azo dye(11,12, 13). On the other hand, auramine does not affect development of liver tumors when fed with the dye(9), yet it was found that addition of this compound in small amounts markedly inhibited dye destruction in the present experiments.

The inhibitory effect of certain benzimidazoles on carcinogenesis(1) would not seem to be related to increased dye destruction, since these compounds tend to preserve the intact dye in liver homogenate systems. On the other hand, the process of dye destruction would seem to be associated in some way with carcinogenesis. Of the benzimidazoles studied to date(1), there is good correlation between anti-azo dye carcinogenic ability and inhibition of azo dye destruction; *i.e.*, the 2-ethyl-5-methyl benzimidazole is more effective than the 2,5-dimethyl-derivative, which is more active than the unsubstituted benzimi-

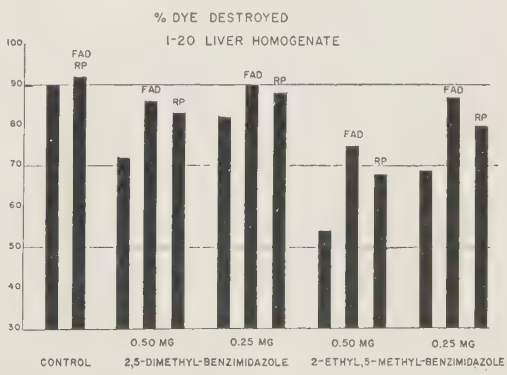

FIG. 1. Effect of flavin adenine dinucleotide (FAD) and riboflavin phosphate (RP) upon destruction of azo dye in the presence of benzimidazoles.

dazole. This same order of activity also exists for inhibition of heme synthesis from glycine by chicken erythrocytes(2,3). The correlation between effect upon heme synthesis and dye destruction, however, is not as good with some of the other benzimidazoles. Certain of the 5-nitro and 5-chloro derivatives are inhibitory in the erythrocyte system(14) while they had little effect upon azo dye destruction. Additional experiments are needed to determine whether anti-cancer properties may be correlated with inhibition of dye destruction or with inhibition in the chicken erythrocyte system. Studies on metabolism of azo dyes with relation to the genesis of liver neoplasia as yet have not revealed the mechanism of the process. Further studies of possible interrelationships between azo dye carcinogenesis, the mechanism of dye destruction in liver homogenates and glycine incorporation in nucleated erythrocytes, might provide some basic information.

Summary. Certain benzimidazole, benzothiazole, indole, and quinoline derivatives, and auramine, inhibit destruction of the azo dye, 4-dimethylaminoazobenzene, by liver homogenates. This inhibition can be partially overcome by addition of riboflavin adenine dinucleotide and to a lesser extent by riboflavin phosphate. Correlation of inhibition of *in vitro* dye destruction and of heme synthesis by avian erythrocytes with the anti-azo dye carcinogenic effect of certain benzimidazoles is discussed.

The technical assistance of Mr. W. Friend is gratefully acknowledged.

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Renal Function in Human Pregnancy. I. Changes in Glomerular Filtration Rate and Renal Plasma Flow* (23790)

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The reports on the effects of pregnancy on renal functions have been controversial. Earlier investigators(1,2,3) have asserted that pregnancy does not alter renal functions while more recent studies(4,5) have shown a progressive increase in renal plasma flow (RPF) and glomerular filtration rate (GFR) which reaches a maximum around the eighth month of gestation and returns to near normal non-pregnant values after gestation. This divergency of opinion is probably due to the fact that the data reported by various authors were obtained from different groups of patients studied at different periods of gestation and were compared to still another group of non-pregnant individuals.

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The present study was aimed at investigating renal plasma flow and glomerular filtration rate in the same individual at various periods of gestation and after delivery. In this way the subject serves as her own control and errors from patient variation are eliminated.

Material and methods. Nine patients with normal pregnancy and without any history of cardiovascular or renal disease were selected. Each patient was studied in the first trimester (12-15 weeks), 2nd trimester (22-28 weeks) and 3rd trimester (33-37 weeks) of pregnancy and 6 to 8 weeks after delivery. The studies were conducted in the post-absorptive state with the patient in the recumbent position. Water diuresis was induced by liberal use of oral fluids just before and during the renal test and by intravenous isotonic glucose solution. A priming dose of inulin and PAH, calculated for each patient according to body weight, was injected intravenously over a period of 2 to 3 minutes and was followed by a sustaining infusion aimed at maintaining plasma levels of 200 mg/l of inulin and 30 mg/l of PAH. An equilibration period of 30 to 45 minutes was allowed after which 4 to 6 clearances of 20 to 30 minutes duration were obtained. Completeness of urine collections was achieved by exerting suprapubic pressure and by injecting air into the bladder. Blood samples were obtained at the midpoint of each collection. Inulin and PAH in blood and urine were analyzed by methods in current use in these laboratories. RPF was estimated from the PAH and GFR from the inulin clearances. It has been shown(4,6) that these substances are as suitable in the pregnant as in the non-pregnant subject to measure RPF and GFR.

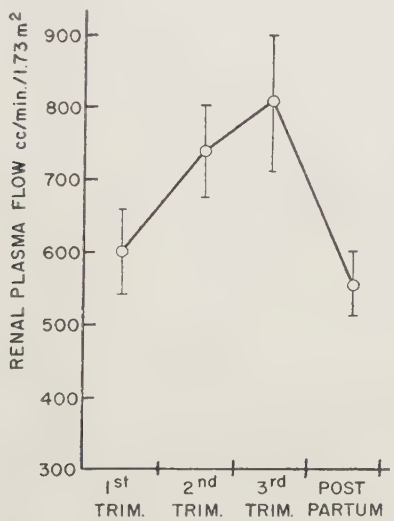

FIG. 1. Changes in renal plasma flow during normal pregnancy and after delivery.

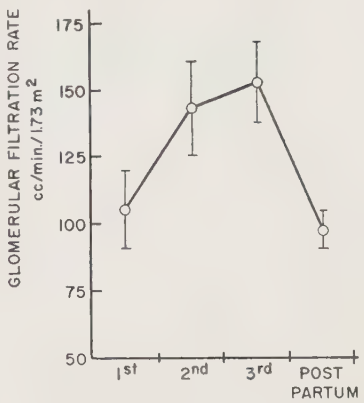

FIG. 2. Changes in glomerular filtration rate during normal pregnancy and after delivery.

Results. Fig. 1 shows average PAH clearances for the 9 patients during the 1st, 2nd, and 3rd trimester of pregnancy and in the postpartum period. The vertical bars represent standard deviation of the mean. Renal plasma flow rose in each patient from the first to the second trimester with a further but a less striking rise between the second and third trimester of pregnancy. In the postpartum period, renal plasma flow fell to values below those observed in the first trimester of pregnancy. Fig. 2 shows the values for the inulin clearances. The pattern of change was the same as that seen with PAH. Marked individual variations in the values for both PAH and inulin clearances occurred and were more evident during pregnancy than in the postpartum period.

Discussion. The present data are in agreement with those of Bonsnes and Lange(5) and Bucht(4) and show a significant rise in both renal plasma flow and glomerular filtration during pregnancy with a return to normal non-pregnant values 6 to 8 weeks after delivery. The rise was already evident in the 1st trimester but it was more striking during the 2nd and 3rd trimester of pregnancy.

There is a common belief among obstetri-

cians that the increased metabolic processes of pregnancy place a burden on the kidneys. The term "low reserve kidney" is frequently mentioned in obstetrics and usually denotes a kidney nearly exhausted by pregnancy. This concept has led many obstetricians to advise against pregnancy in the presence of any renal abnormality. In view of the increase in renal circulation and in glomerular filtration rate in pregnancy, this concept seems to be untenable.

Summary. 1. Renal function studies were performed on 9 patients during 1st, 2nd and 3rd trimester of pregnancy and in the postpartum period. 2. A progressive increase in renal plasma flow and glomerular filtration occurred, reaching maximum in 3rd trimester and returning to non-pregnant levels after delivery. 3. These changes speak against an added burden imposed on the kidneys by pregnancy.

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Effects of Superimposed Native Insulin on Disposal of Iodoinsulin in the Body.*† (23791)

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Iodoinsulin is widely used as a tracer in work designed to study the physiology of insulin. It is assumed that the iodinated compound is treated like native insulin by the tissues of the body. The validity of this assumption is supported by the observation that the breakdown of iodoinsulin in the body can be slowed by simultaneous administration of large doses of native insulin(1). This suggests the degrading processes of the two compounds are related and that some at least of the degrading mechanisms do not distinguish between them. Additional support for this assumption was given by our finding that biological activity resulting from a dose of native insulin decayed at the same rate as labeled insulin given at the same time to eviscerated-nephrectomized rabbits(2). In the intact animal the decay rate of iodoinsulin is very rapid. Greeley found the decay of biological activity to be much slower(3). There are many possible causes for this discrepancy. The kidney and liver, which actively degrade iodoinsulin, may treat native insulin differently. Another possible cause of the discrepancy is that iodoinsulin is ordinarily used in tracer amounts whereas appreciable dosages of insulin must be given to follow biological activity. Large doses of native insulin definitely lower the decay rate of iodoinsulin when the two are given together(1). It is possible that the smaller doses used to study biological activity could also produce such an effect. The experiments reported in this paper were planned to study this factor. The decay of iodoinsulin in the intact animal is so rapid that it would be difficult to detect small changes that might be effected by moderate doses of native insulin. Very large doses of

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† The crystalline zinc insulin used was contributed by Lilly Research Laboratories.

native insulin are needed to give enough effect to be detected. The decay rate is very much lower in eviscerated-nephrectomized rabbits and we made use of this preparation with the view that much smaller effects could be detected with it. Several animals were so prepared. Each was given a tracer dose of iodoinsulin. In addition some were given simultaneously a dose of native insulin which varied over a wide range.

Methods. Insulin-I^{131} Degradation Assay. The insulin-I^{131} was purchased from Abbott Laboratories. The degradation of insulin-I^{131} was measured by the rise in radioactivity in the 10% trichloracetic acid (TCA) soluble fraction. This is based on the fact that the radioactivity of insulin-I^{131} is at least 95% precipitable with TCA, and it is assumed that the non-precipitable radioactivity represents breakdown products of the insulin-I^{131} molecule. Accordingly, the I^{131} content of the plasma and of the TCA soluble plasma fraction were determined. All radioactivity measurements were made in a well-type scintillation counter. All glassware used in the transfer of the insulin-I^{131} was washed with 10% NaOH and assayed for its radioactivity content so that corrections for the amount of radioactivity injected into the animals could be made. In no experiment did the I^{131} absorption by the glassware exceed 2% of the anticipated injected radioactivity. The rabbits were eviscerated by the method previously described(4). Native and iodoinsulin were mixed in about 4 ml saline which was injected intravenously followed by 3 rinses of 2 ml saline each. The total volume injected amounted to about 5 ml per kilo body weight. Volume of distribution of iodoinsulin was calculated by dividing the radioactivity per ml plasma into the total activity given. The plasma radioactivity includes both the TCA-soluble and precipitable fractions. We also calculated the volume of distribution after correcting for the estimated TCA-soluble activity. Since the degraded iodine appears to be largely associated with quite small molecules (5) it might rapidly distribute in the extracellular compartment. We estimated the total activity of the "degraded" iodine by multiplying the TCA-soluble activity per ml plasma by the estimated volume of the extracellular compartment (25% of body weight). Such a correction did not change the results significantly.

TABLE I. Effect of Superimposed Doses of Native Insulin on Degradation of Iodoinsulin at Various Times after Injection in Eviscerated-Nephrectomized Rabbits. Figures represent % of total plasma activity that is TCA soluble.

	Time after inj. in min.			
Units insulin	15	30	60	120
Tracer*	11	19	34	56
1.4		10	18	28
1.42		15		40
1.65	6	13	24	39
1.7	9	14	21	37
10	10	13	21	27
100	6	9	15	26
200	3	5	11	26
500	2	4	8	19
1000	2	3	12	27

* Tracer alone; avg of 9 animals.

Results. Table I shows the effects of superimposing a dose of native insulin on the rate of decay of iodoinsulin in the eviscerated-nephrectomized rabbit. As to be expected, very large doses definitely delayed the breakdown of the labeled compound. Much smaller doses (10 down to 1.4 units per kg) also had an appreciable effect. These intermediate doses are in the range of those ordinarily used to follow biological activity.

While carrying out these experiments we made the incidental finding that superimposed native insulin delays the apparent distribution of iodoinsulin in the body. In the eviscerated-nephrectomized animal tracer doses of iodoinsulin appear to distribute in a volume somewhat more than that of the extracellular compartment(2). The results shown in Table II might suggest that the native insulin slowed up the passage of iodoinsulin from the intravascular, to the interstitial, compartment and reduced the final volume of distribution. However another explanation is more likely. Some special tissue may take up iodoinsulin and hold it by some process that could become saturated if the concentration of the hormone were high. This tissue could not distinguish between native and labeled insulin so then carrier insulin would retard the passage of the labeled compound from the blood stream. This sequestration cannot be very large since

TABLE II. Effect of Superimposed Doses of Native Insulin on Apparent Volume of Distribution of Iodoinsulin at Various Times after Injection in Eviscerated-Nephrectomized Rabbits. Figures represent calculated volume of distribution of iodoinsulin expressed as ml/100 g body wt.

	Time after inj. in min.			
Units insulin	15	30	60	120
Tracer*	14	21	29	32
10	14	17	21	24
100	9	12	17	20
200	11	16	21	22
500	6	11	16	20
1000	7	10	16	20

* Tracer alone; avg of 8 animals.

the apparent volumes of distribution of labeled insulin with and without carrier are 20 and 30% of body volume respectively at the end of 2 hours. This concept is supported by the fact that the volume with carrier is close to that of the extracellular space. A compound with the relatively low molecular weight of iodoinsulin must at least distribute in the extracellular compartment.

Discussion. Our results explain in part why insulin action as measured by its effect on sugar metabolism seems to indicate a decay rate of the hormone in the body much less than that of iodoinsulin as measured by conventional methods. To measure biological activity, one must use much larger doses of insulin than workers ordinarily use in studying the fate of iodoinsulin in the body. On the basis of our findings we believe that moderate doses of native insulin must depress the breakdown of the labeled compound in the intact animal although this would be difficult to demonstrate because of the rapid degradation and the high variability between animals. However this factor cannot entirely account for the discrepancy. Insulin binding by specific plasma globulin produced as a result of previous insulin injections(5) could have prolonged the biological activity of the hormone in the animals studied by Greeley. These dogs were pancreatectomized in order to eliminate the variability of endogenous insulin and had to be maintained on insulin. To what extent insulin binding could have affected the results would have to be determined by comparing the biological effects of doses of insulin given soon after pancreatectomy and some weeks later in the same animal.

The effect of large doses of native insulin in reducing the apparent volume of distribution of iodoinsulin is most likely the result of a mechanism similar to that acting on the degradation process. Our results suggest that when iodoinsulin is given in tracer doses there is sequestration of a portion of it by some process that can be saturated by a large dose of native insulin. Experiments designed to study the mixing of iodoinsulin in the body and its ultimate volume of distribution should be carried out with the addition of a large amount of native insulin. The values for these which we give in this paper should be more correct than those given in our earlier publication(2).

It would follow from our findings that when iodoinsulin is used as an indicator of the behavior of natural insulin in the body, the latter should be added in physiological amounts, or in larger dosages if the investigator is concerned with the disposal of such.

Summary. In eviscerated-nephrectomized rabbits the decay of iodoinsulin is retarded by addition of moderate and physiological doses of native insulin. The apparent volume of distribution of iodoinsulin is reduced by superimposed native insulin. This suggests a sequestering of part of the tracer compound by a process that can be saturated by added native insulin. The results indicate that in studying the physiology of insulin by use of iodoinsulin, investigators should add native insulin in appropriate dosage. Determinations carried out in this way indicate that iodoinsulin distributes in the eviscerated-nephrectomized rabbit in a volume equal to the extracellular compartment.

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Relation of Lymph to Distending Fluids of the Kidney.* (23792)

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When the renal artery and vein are simultaneously clamped and then the vein cut and the organ allowed to drain, there flows out a fluid, to be designated as "kidney fluid," which differs considerably from systemic blood in composition. Its red cell content is only half that of blood(1,2). This fact has lead to the hypothesis that renal vascular blood, under the conditions of this experiment, is diluted with another fluid. The latter is designated as "red cell-diluting fluid," or, more simply, "diluting fluid." This term, along with the term "kidney fluid," will be frequently used in the present paper. "Kidney fluid" also differs from systemic blood in that its plasma is relatively high in K, Cl, PO_4 and urea, but low in glucose, inulin and protein(1). "Diluting fluid," it is therefore postulated, is not only red cell-free, but also differs from blood plasma considerably. Its origin is unknown. But we have postulated that it comes from a large interstitial compartment(1,3), basing the hypothesis in part on the similarities in composition of "diluting fluid" and renal lymph as reported by Sugarman, Friedman, Barrett and Addis(4) and Kaplan, Friedman and Kruger(5). A more extensive analysis of the composition of the various fluids is reported here: arterial blood, venous blood, renal lymph, urine, and the kidney's distending fluids. Because lymph is generally considered to resemble organ interstitial fluids closely(6), lymph analysis should give clues as to the nature of the kidney's postulated interstitial fluids. It is also known that as fluid drains from the functionally distended kidney, its content of red cells progressively decreases until the last portion to drain shows a red cell content of about 7%(1). It was thought that this sample should most closely resemble renal lymph in composition, if "diluting fluid" were indeed interstitial fluid, and hence this sample was analyzed separately from the major sample draining. Finally, quantitative measurements of the several plasma proteins in the various fluids were made, because this information might also furnish clues as to the nature of "diluting fluid."

Methods. Dogs were anesthetized with Pentothal,† 25 mg/kg, followed with barbital-sodium, 200 mg/kg. Capsular lymphatics served as a source of lymph; these, it is generally considered, drain primarily from the cortex, but in some dogs from the medulla as well(4,7,8,9). After exposing the kidney through a flank incision, the mass of tissue surrounding the posterior pole was tied, blocking lymph drainage at this point and causing capsular lymphatics to swell. Into one of these was inserted a fine, polyethylene catheter (O. D. 0.024 in.), and free drainage allowed. Lymph flow was slow, at a rate of 0.1-0.3 ml/hr (Table III). Since 0.4-0.8 ml was collected, this phase of the experiment took 2-4 hrs. During it, lymphatics of the anterior pole and of the hilus remained undisturbed. Also during this period, urine was collected from a catheter inserted into the homolateral ureter. No diuretics were administered. After the lymph sample was collected, the dog was suspended in the prone position and the kidney dissected free from all tissue except artery, vein and ureter. Subsequent samples were taken as previously described(1): first, blood samples from the carotid artery and from the renal vein while the organ was still functioning. Then hilar blood vessels were doubly clamped, the distended kidney removed and placed in a beaker, blood vessels cut, and the organ allowed to drain for 1 minute. In volume this sample comprises about 75% of the fluids which functionally distend the kidney; it will

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† Kindly furnished by Abbott Laboratories, N. Chicago, Ill.

TABLE I. Composition of the Several Fluids.

	A*	B*	C*	D	E	F	G	H
	Renal venous blood	Lymph	Urine	Artery Vein	Kidney fluid I Vein	Kidney fluid II Vein	Kidney fluid II Lymph	Diluting fluid Lymph
Hematocrit	38			39/38	22/38	7/38		
Plasma: Na	148	140	132	.97	.99	.98	1.03	1.03
K	4.5	5.9	183	1.04	1.22	2.26	1.73	1.86
Cl	120	129	64	1.05	1.07	1.10	1.01	1.00
PO_4 as P	4.7	5.3	322	1.13	1.81	1.76	1.56	1.66
Glucose	109	28	0	1.00	.84	.23	.90	.57
Urea	33	46	2050	1.00	1.60	1.95	1.40	1.47
Protein	6.4	3.2		1.05	.69	.50	1.02	.98

* The units employed in these columns are as follows: Na, K and Cl, in meq/l; PO_4 as P, glucose and urea, in mg %; protein, in g %.

be designated as Kidney Fluid I. The kidney was then transferred to a second beaker and allowed to drain for 15 minutes more. This last sample was about 3 ml in volume; it is designated as Kidney Fluid II. Heparin was used as anticoagulant. Routinely, microhematocrits were taken. Plasmas of large samples (arterial and venous bloods and Kidney Fluid I) and urine were analyzed for Na, K, Cl, PO_4 (as P), glucose, urea and protein by methods previously described(1). In the case of small samples (plasma of Kidney Fluid II and lymph), Na and K were measured by flame photometry, and Cl, glucose, urea and protein by the micromethods of Natelson(10). Inorganic phosphate was determined by the improved quinaldine-red method of Soyenkoff(11). Plasma protein patterns were obtained by paper electrophoresis, stained with bromphenol blue, and the intensity of the spots recorded by means of a densitometer (Photovolt Model 501A). Areas under the curves, measured by planimeter, were considered proportional to the amount of protein. Three fractions were measured: (1) albumin and α-1 globulin. (2) β-globulin and α-2 globulin and (3) γ-globulin.

Results. In Table I are shown, in Columns A, B, and C, averaged results for five dogs for renal venous blood, lymph and urine respectively. In subsequent columns are shown ratios of the contents of various substances in various fluids; from these ratios the raw data may readily be deduced. The table confirms a number of conclusions already drawn in a previous report(1): 1) as blood flows through the kidney, it changes only minimally in composition (Column D); 2) the first three-quarters of fluid to drain from the kidney (Kidney Fluid I) has a hematocrit less than that of blood (Column E) and the last quarter relatively few red cells (Column F); 3) plasma of "kidney fluid" is richer than blood plasma in K, Cl, PO_4 and urea, but poorer in glucose and protein (Columns E and F). The table also presents in Column G the composition of the last quarter of "kidney fluid" to drain (Kidney Fluid II) relative to that of lymph. This part of the kidney's distending fluid has the same content as lymph of Na, Cl, glucose, and protein, somewhat more urea and PO_4, and clearly more K. In Column H of the table are shown ratios of calculated content of various substances in "diluting fluid" to those in lymph. Methods used for the calculation have been previously described (1). Data for Kidney Fluid II and for renal venous blood were used. The ratios found are

TABLE II. Plasma Proteins in the Several Fluids.

	Artery	Renal vein	Kidney fluid I	Kidney fluid II	Lymph
Total protein, g %	6.7	6.4	4.4	3.2	3.2
Albumin and α-1 globulin, %	30	30	28	29	36
β-globulin and α-2 globulin, "	42	44	43	42	42
γ-globulin, "	28	26	29	29	22

TABLE III. Data on 5 Dogs.

Lymph flow, ml/hr	Protein			K, meq/l			Cl, meq/l		
	Lymph, g %	Lymph / Vein	Lymph / Kidney fluid II	Lymph	Kidney fluid II	Urine	Lymph	Kidney fluid II	Urine
.09	2.9	.56	.97	7.2	9.5	119	125	129	64
.31	2.7	.40	1.00	4.5	10.1	232	131	132	98
.11	2.2	.34	.82	6.3	11.4	198	136	134	89
.25	5.8	.83	1.07	5.8	9.6	136	132	138	38
.35	2.5	.38	1.04	5.9	10.6	228	122	123	30
Avg									
.22	3.2	.50	.98	5.9	10.2	183	129	131	64

in general close to those of Column G.

Table II indicates the relative proportions of plasma proteins in the several fluids. Both lymph and Kidney Fluid II had the same quantity of protein, *i.e.* one-half that of plasma. In both samples of "kidney fluid" and in arterial and venous plasma, the fractional distribution of protein between the 3 moieties was the same. Lymph had, proportionately, slightly more albumin and slightly less gamma-globulin than did plasma.

Table III shows for each of 5 dogs rates of lymph flow and their individual protein contents. This is of interest because of the report of Sugarman *et al.*(4) that protein content of renal lymph varies inversely with its rate of flow. In our experience, as the table shows, this was not apparent: lymph protein content was about the same for all dogs, in spite of flow variation. The table also shows ratios of lymph protein content to venous plasma content and to Kidney Fluid II content: lymph has half the quantity of protein that venous plasma has, but almost exactly the same quantity of protein as Kidney Fluid II.

In a previous paper, it was reported that "kidney fluid" was not related to urine in any consistent way(1). The same conclusion may be drawn for renal lymph (with confirmation for "kidney fluid") from Columns B and C of Table I: lymph, for example, is high in its Cl content whereas urine is low. And urine has a very high K, PO_4 and urea content, whereas lymph content of the 3 is only slightly higher than that of blood plasma. In the previous paper it was also pointed out that even though different dogs were forming urines of considerably different composition, this did not affect the composition of "kidney fluid." The same conclusion applies to kidney lymph; illustrative support for K and Cl is given in Table III. Although each dog was excreting the 2 ions at different rates, this individual variability in urinary content is not matched at all by variability in lymph content or Kidney Fluid II content. Instead, both of the latter fluids show a quite constant amount of the 2 ions.

Discussion. Our analysis of renal lymph confirms that of Sugarman *et al.*(4) and Kaplan *et al.*(5) with respect to its urea and sugar content: higher than plasma for urea and lower for sugar. But it differs from that of Sugarman *et al.* in that we found the protein content of lymph to be quite constant at about 3.2 g % (Table III). We also failed to observe the inverse relation of protein content to lymph flow reported by Sugarman *et al.* However, in our experiment only very slow lymph flows were involved; at these low flows, Sugarman *et al.* also found the lymph protein content to be about 3 g %.

Kidney lymph, from this analysis, is fairly similar to cervical lymph(12): higher than blood plasma in its content of K, Cl and PO_4 but lower in its content of protein. The two, in fact, have the same content of protein: half that of plasma. However, it is thought that renal lymph is formed in a fashion far different from cervical lymph. We postulate that renal lymph and the "diluting fluid" have a common origin: they both are a mixture of plasma and tubular resorbate. The theory was originally anticipated by Ludwig(13) and support for it has been obtained by Sugarman *et al.*(4), Kaplan *et al.*(5), and Foldi

and Romhanyi(8).

Before considering this hypothesis, two others, both of which we think erroneous, will be discussed. We have previously argued(1) that "diluting fluid" does not originate in any way from tubular urine because: 1) its composition is conspicuously different from that of the tubular contents; 2) its composition is quite constant relative to blood and does not vary from dog to dog, as does that of urine; and 3) its content of protein remains at about 3 g % as successive fractions drain, whereas this would not occur if successive fractions contained more and more tubular urine. All of these arguments can equally well be applied against the theory that "diluting fluid" is proximal tubular urine which, under the conditions of the experiment, reenters the vascular system by passing backwards through the glomerular endothelium. In addition, if such reentry occurred, "diluting fluid" should be very rich in inulin and diodrast, because proximal tubular urine is also (presumably) very rich in them. But this is known not to be so (1). Finally, the hypothesis that "diluting fluid" originates in part from cell juices, due perhaps to anoxia, has also been discussed and tentatively dismissed(1).

Instead, we postulate that "diluting fluid" and renal lymph are both a mixture of plasma and tubular resorbate. It is thought that plasma penetrates the peritubular capillary endothelium with utmost freedom (see below), and then in the interstitial space it is admixed with a large volume of tubular resorbate which is in transit toward capillary lumina. At the instant, in the present experiment, when hilar blood vessels are clamped, the interstitial space is ballooned out with resorbate and blood plasma; these fluids then, under pressure from the elastic distended kidney, are pushed into the most accessible channel: they run easily back through the porous endothelium and thence out of the renal venous system mixed in turn with vascular blood. In summary, the sources of "kidney fluid" are postulated to be these: 1) vascular blood 2) plasma filtered from blood, and 3) tubular resorbate. The last 2 of these 3 comprise what we designate as "diluting fluid." A preliminary estimate of the volume of tubular resorbate may be made from the protein content of "diluting fluid": it is roughly the same as that of the filtered plasma, and the 2 together comprise about half of the "kidney fluid."

Furthermore, if one makes the ancillary hypothesis, justification for which will be presented below, that the resorbate involved comes primarily from the proximal tubules, one can explain in part the unusual composition of "kidney fluid." Proximal tubular resorbate might be expected to have, in comparison with blood plasma, the following composition: the same amount of Na, more K (14), and little or no protein. Such a fluid as this, mixed with vascular plasma, resembles the observed "kidney fluid" and lymph fairly closely. However, the theory fails to account for the concentration of three substances in "kidney fluid": urea, Cl and sugar. One would expect both urea and Cl, particularly the latter(15), to be low in resorbate and hence low in "kidney fluid." One would also expect that the sugar content of proximal resorbate, and therefore of "kidney fluid," would be about the same as that of blood, but this is not so. The kidney's ability to manufacture sugar complicates the matter greatly (16).

In support of the hypothesis that the resorbate comes primarily from the proximal convolutions of the nephron, it is known that proximal resorbate is much greater in volume than that from the distal convolutions, being, in obligative resorption, some 4 times greater than resorption from other regions(17). *Pari passu,* the volume of the proximal convolutions, as calculated from dimensions given by Maximov and Blood(18) for human kidneys, is some 8 times greater than that of the distal convolutions. Finally, the composition of proximal resorbate is generally thought to be constant, and not to vary as does urine composition, a characteristic shared by "kidney fluid."

The presence of protein molecules in "kidney fluid" and renal lymph in the same proportions as in blood plasma indicates, following Grotte's recent hypothesis(19), that very large "leaks" must be present in the peritubular capillary endothelium. These are thought to be much bigger than the pores in

which molecular seiving of different sizes of protein molecules might occur. Grotte was able to give a preliminary estimate of the size of "leaks" in leg and liver capillaries: they must be greater in diameter than 232 Ångstroms, but smaller than 700. In peritubular capillaries holes of this size are also apparently present: Pease(20) finds numerous pores in the thin endothelium from 400 to 500 Å wide. Using Pease' microphotographs, we estimate (with due caution) that the peritubular capillary wall has roughly 40 to 120 "leaks" per square micron of surface, with a combined area of 5 to 25% of wall area. It is through this porous membrane—indeed, it is more like a collander—that plasma is postulated to run so freely. There appears, thus, to be good morphological data in support of the experiments and hypotheses here reported.

These deductions fit well into our original hypothesis concerning the peculiar renal circulation(1). As we now view it, peritubular vascular blood runs in channels bounded by a membrane containing numerous large leaks some 500Å in diameter. These prevent passage of red cells into interstitial fluid, but they allow all plasma constituents to enter with complete freedom. Thus a specialized plasma circulation to the tubular portions of the nephron is furnished. Plasma, simultaneously diluted with incoming resorbate, is thought eventually to run into a profuse system of microcanaliculi which penetrate deep into each cell and which are separated from cell constituents only by delicate invaginated plasma membranes(21,1).‡ A morphological intimacy like this is, indeed, almost a prerequisite for the completeness of the kidney's extraction from blood plasma of substances like p-amino-hippuric acid.

This theory of renal circulation is to be contrasted with that of Pappenheimer and Kinter, who suggest that plasma is twice skimmed off from red cells, first at inner afferent arterioles (*i.e.* those closer to the medulla), and second at the start of the peritubular capillary bed(22). The volume of the plasma-rich channels is thought to be larger than that of red cell channels. The data on composition of "kidney fluid," particularly in protein, oppose this hypothesis. We consider that plasma skimming, in the conventional sense of the term, does not occur in the discrete vascular system of the kidney. Rather, in peritubular capillaries, the porous endothelium "seines out" red cells and allows a large volume of plasma to enter the interstitial space and to come into utmost intimacy with tubular cells.

Summary. Renal lymph from dogs was analyzed, along with the fluids which naturally distend the kidney, arterial and renal venous blood, and urine. Lymph resembled the last quarter of the kidney's drained fluid in its content of Na, Cl, glucose and protein, but had less urea, K and PO_4. Both fluids had half the protein content of blood plasma, but the same fractional distribution, measured by paper electrophoresis, of albumin and α-1 globulin, β-globulin and α-2 globulin, and γ-globulin. Both fluids were also observed to have a constant composition in the face of varying urinary composition. The hypothesis is developed that fluids which functionally distend the kidney and which drain out of the vein when the artery is clamped originate from two sources: vascular blood and a large interstitial compartment. It is further postulated that blood plasma enters the interstitial compartment with utmost freedom. This compartment in turn is functionally distended with plasma plus resorbate in transit from tubules to the vascular system.

‡ The theory assumes that the basement membrane presents no barrier to passage of solvents or solutes.

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Microcirculatory Changes in the Liver of Choline-Deficient Rats.*† (23793)

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The promptness with which lesions occur in zone 3 of the "liver acinus" of a rat even after a short period of choline deficiency(8) made us think that a circulatory factor must be playing its part in this disorder. By "liver acinus" we mean the structural and functional hepatic unit(1-2), the concept of which is based on the dependence of a microscopic amount of hepatic parenchyma on the terminal afferent branches bringing in materials for metabolism and on the associated terminal bile ductule carrying away the secretory product, the bile. Zones can be outlined in the acinus, areas of distinct enzymatic patterns(3), close to, or remote from the source of supply of oxygen and nutrients, *i.e.,* the terminal afferent vascular branches. One can easily see (Fig. 1) that it is zone 3 of the acinus farthest from the terminal portal and hepatic arterial branches that shows the fatty change first. The following method was adopted to observe the early microcirculatory changes in choline-deficient livers.

Method. Fifteen weanling rats (male and female) weighing between 60-80 g were fed a severely choline-deficient diet for periods ranging from 12 hours to 7 days. The diet contained 20% mixed proteins, was low in methionine and supplemented with cystine to make the organic sulfur adequate. The intrahepatic circulation was studied *in vivo* in Dr. Knisely's laboratory using his transillumination technic with the fused quartz rod(4). A technic was developed to immobilize the liver and to observe its circulation under physiologic conditions in laparotomized rats. A polyethylene apron (Fig. 2) cut to the width and length of the abdominal cavity is slipped with its folded upper margin beneath the liver. This apron has a fine hole in the lower midline through which is passed a polyethylene tubing flanged at one end. The tubing is then connected with a container of warm Ringer solution. Inflow and pressure of the solution are adjusted by a micrometer screw. This device made it possible to control the pressure gradient between abdominal and thoracic cavity. The pressure in the latter was raised by the insufflation of O_2 under 40-50 mm Hg *via* a tracheal cannula in order to immobilize diaphragm and liver(5). The insufflation pressure was controlled by a needle valve. However, the increase in insufflation pressure required to immobilize these structures was 1/6

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to 1/10 of that needed when no abdominal apron was used to splint the diaphragm. In this preparation the sinusoidal circulation was continuous and swift, and it was possible to study the same field with the water immersion lens for hours.

Results. Six rats in choline deficiency from 24 to 36 hours showed definite changes in hepatic parenchyma and circulation. The liver edge was more transparent and contained tiny fat globules in the hepatic parenchymal

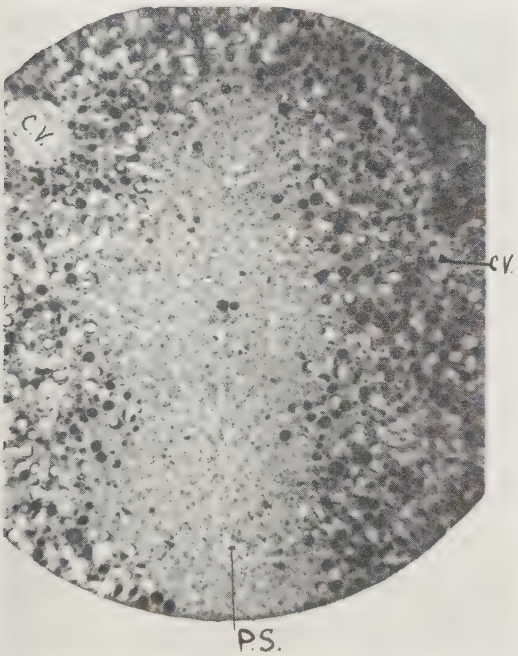

FIG. 1. Liver of a rat fed a low choline diet. One can recognize the fat droplets (black) in cells of zone 3 which lies concentrically around terminal afferent vessels branching out from a portal space (P.S.). The central veins (C.V.) at the periphery of the liver acinus are linked to each other by the band of fatty tissue arching around the tip of the acinus (90 × approx.).

cells, smaller and more regular than those seen in the mesentery. The sinusoidal pattern was smudged as if somebody had passed a greasy hand over the lenses. The central veins stood out in a field of less detail. Many of the outlet sphincters at the junction of sinusoids and hepatic veins were closed, and the liver seemed to be in a prolonged state of what Knisely termed "storage phase"(6-7). The tiny globules seen with the water immersion lens were single, or in rows, and adjoined

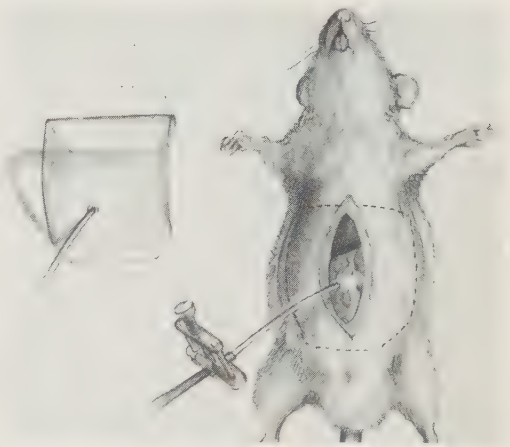

FIG. 2. Polyethylene apron covers viscera of a laparotomized rat. Ringer solution is introduced beneath the plastic sheet into peritoneal cavity raising intra-abdominal pressure.

the hepatic sinusoids (Fig. 3). Some overlay the sinusoids and swayed when the blood passed beneath them. When a coverglass was pressed slightly on the area, the tiny droplets produced pits in the tissue; on removal of the coverglass the pits disappeared and the droplets became prominent again. Thus we demonstrated that the fat droplets are less compressible than the parenchyma which yields to pressure which they exert. In one field such

FIG. 3. Drawing of a rat liver transilluminated and observed *in vivo*. The animal was on a severely choline deficient diet for 36 hr. For description see text (× 225).

a globule overlay an arterialized sinusoid and pulsated back and forth with the onrushing and receding pulse wave beneath it. It was obvious at this magnification that the fat droplets definitely pressed on the sinusoids and impeded the passage of blood. When after hours of observation the continuous flow had slowed and become granular in character one could see red cells squeezing between opposing fat globules.

In 3 other rats studied after 48-96 hours of choline deficiency the changes were more accentuated. One had the impression that the tissue had been strewn with round transparent pellets that covered and distorted the sinusoids making the flow harder to follow. The central veins although slender still showed good flow coming from the deeper layers.

A group of 6 rats on a choline deficient diet for 5 to 7 days were studied by the same technic. Under low magnification (X 50) the sinusoidal pattern had almost faded. It could be made to reappear by raising the insufflation pressure of the O_2 to about 60 mm Hg. This pressure impeded the outflow of blood from the hepatic veins and the blood stagnated, dilating the hepatic sinusoids. Under medium magnification (X 100) the hepatic cells appeared fatty and some looked as if they were translucent bubbles. Rows of small globules were tightly packed against the sinusoidal walls. The red streaks representing the flowing blood in the sinusoids were narrow and had a conspicuously indented outline due to the compression of the sinusoidal walls by the fat globules. The smaller the globules the less compressible they were.

Summary. (1) A method has been developed by which intraabdominal pressure sufficient to immobilize the liver can be established in small animals laparotomized for the study of the intrahepatic circulation *in vivo*. (2) In choline-deficient rats there is an accumulation of tiny fat droplets along the sinusoidal walls or overlying them as early as 24 or 36 hours after the ingestion of the deficient diet. The small and barely compressible droplets exert a pressure on the pliable endothelial walls indenting them and impeding the sinusoidal flow. (3) These phenomena are conspicuous on the surface of the transilluminated liver edge which of course is formed by zones 3 of the acini under observation.

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The Penn Seroflocculation Reaction in Cancer Diagnosis.* (23794)

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A seroflocculation reaction was reported by Hall, Dowdy, Penn, and Bellamy(1) to have significant cancer selectivity, *i.e.*, in testing over 10,000 sera their correct positive results in the cancer patients ranged between 81 and 98%. Their false positive results in testing patients ranged between 9 and 33% while their false positive results in testing normal persons ranged between 0 and 4%. These results were obtained using 3 different "anti-

* This investigation was supported by research grant from Nat. Cancer Inst.

gens" (A, B, & C) over a period of 10 years. Using a still different "antigen" (D), presumably ethyl choladienate, they found 100% correct positive results in testing 80 cancer sera, 57% false positive results in testing 85 non-cancer sera, and 3.8% false positive results in testing sera from 103 normal individuals. At the invitation of Dr. Dowdy, several laboratories in different parts of the country undertook a group study of the Penn seroflocculation reaction using ethyl choladienate as antigen. Our participation consisted of a clinical trial of the reaction using sera from patients in the University of Kansas Medical Center. This report describes the results obtained in testing 241 cancerous and 1,608 non-cancerous patients as well as 129 normal ambulatory individuals. Although the positive results in the normal group were only 5% and the 12% positive results in the non-cancer group were not excessive, the procedure was not a satisfactory cancer test since it was positive in only 43% of the cancer group.

Method. The seroflocculation procedure is based on the reaction between serum and an "antigen" composed of buffered saline, cholesterol and ethyl choladienate. The qualitative test which is routinely performed uses 2 different ratios of serum to antigen. A positive reaction in either tube is considered indicative of cancer and is recorded when aggregates form and the liquid phase becomes less turbid. Varying degrees of turbidity without particle formation are recorded as negative results. Instructions and practice in performing the test for staff from this laboratory were kindly provided in the laboratories of Hall and Penn. The method was that published for "antigen" D by Penn and Hokama(2). The antigen was ethyl choladienate supplied to us by Hall. The seroflocculation test light recommended by Hall was used in estimating the degree of aggregation in the tubes. All sera were obtained from patients in the University of Kansas Medical Center and were tested without knowledge of the diagnosis. Classification of the patients was carried out several months later after careful review of the completed clinical record. The test results and the clinical classification data were forwarded to Dr. J. E. Dunn, Jr. of the U.S. Public Health Service in Berkeley, Calif., for analysis and correlation with similar data produced by the other laboratories. The incidence of the various types of nonmalignant disease is shown in Table I. The numbers of patients tested in each category are shown in Part A of Table I, while Part B gives the numbers after deletion of certain classes of patients known to have a high incidence of false positive results, *i.e.*, those having hay fever or asthma, rheumatism or arthritis, active tu-

TABLE I. Incidence of Positive Results in the Various Types of Non-Malignant Disease and in the Normal Group.

	A. Without deletion			B. With deletion of certain patients*		
Type of disease	No. of patients	No. & % patients with positive results		No. of patients	No. & % patients with positive results	
Minimal disease	313	17	5	312	17	5
Types of inflammation	303	98	32	189	33	12
Heart disease	243	51	21	211	32	15
Types of hyperplasia	121	15	12	117	14	12
Ophthalmic cases	119	16	13	109	12	11
Benign tumor	84	9	11	80	6	8
Diabetes mellitus	74	19	26	70	17	24
Pregnancy	71	28	39	0	0	
Gastric & duodenal ulcer	51	12	24	45	9	20
Miscellaneous	229	42	18	206	29	14
Total	1608	307	19	1339	169	12
Normals	129	6	5			

* Deleted were all patients with allergy, arthritis, acute tuberculosis or syphilis, temp. over 99.6°F, and those with trauma, surgery or blood transfusion in past 10 days as recommended by Hall.

TABLE II. Incidence of Positive Results in the Untreated Cancer Group* According to Type or Site of Tumor.

Type or site of tumor	No. of patients	No. & % patients with positive results	
Gastro-intestinal tract	50	26	52
Cervix-uteri	31	10	32
Lung	27	13	48
Prostate	19	5	26
Leukemia	19	6	32
Breast	14	3	21
Bladder	14	5	36
Skin	6	4	67
Pancreas	9	5	56
Kidney	7	2	30
Malignant lymphoma	6	4	67
Miscellaneous	39	13	33
Total	241	96	43

* Without deletion of certain classes of patients as noted in Table I.

berculosis or syphilis, oral temperature over 99.6°F, or trauma, surgery, or blood transfusion in the previous 10 days. Omission of these patients was upon Hall's recommendation. The incidence of the type or site of the cancer is shown in Table II. The tumor was localized in 85 of the 241 cancer patients, regional node involvement or extensive local spread had occurred in 88 and distant metastases were present in 68. All of the cancers were proven by tissue examination. The normal sera were from donors accepted by the hospital blood bank. In the nonmalignant disease group were 711 men and 897 women whose average ages were 58 and 44 years respectively. The average ages of the 88 normal men and of the 41 normal women were 44 and 42 years respectively. There were 123 men and 118 women in the cancer group with respective average ages of 62 and 58 years. Most patients in the minimal disease group had psychoneurotic complaints or were to have minor surgery. Patients in the inflammation group suffered from various diseases in which the primary tissue change was one of inflammation and included those who had recently experienced surgery, accidents or burns. The majority of the patients in the heart disease group suffered from arteriosclerotic heart disease. Most patients in the hyperplasia group had benign prostatic hypertrophy. The patients in the ophthalmic disease group suffered usually from cataracts, glaucoma, or a detached retina. The most common benign tumor was uterine leiomyoma. The patients with diabetes mellitus frequently had arteriosclerotic heart disease and types of inflammation as complications. Most of the pregnancy patients were at term and delivered within the 24 hours preceding the test.

Results. Normal individuals. As shown in Table I only 5% false positive results were obtained in the group of 129 normal ambulatory persons.

Non-cancerous disease group. The incidence of positive results in this group was 19% if all non-cancerous patients were counted (Part A of Table I). This was reduced to 12% (Part B of Table I) when patients having certain disease states were deleted as recommended by Hall. The greatest reduction in false positive results produced by this maneuver (20%) was in the group of patients classified as having some type of inflammation. The effect in the other groups of deleting these patients was slight except in the pregnancy group. The highest incidence of false positive results was in the pregnancy group (39%) and the next highest in the inflammatory disease group (32%) (Part A of Table I). When the deletions recommended by Hall were made (Part B of Table I) there were no pregnancy patients and the highest incidence of false positive results was in the diabetes mellitus group (24%) followed by the gastro-duodenal ulcer group (20%). The group of patients having minimal evidence of organic disease had only 5% false positive results, a figure identical with that obtained in the group of normal ambulatory persons.

Cancerous group. As shown in Table II there were 43% correct positive results in testing the 241 cancer patients if none were deleted because of allergy, arthritis, etc. Although 2 of the various types or sites of cancer (skin and malignant lymphoma) had 67% correct positive results, they contained too few patients(6) for much importance to be attached to this high percentage figure. The next highest incidence of correct positive results was 52% in the gastrointestinal cancer

group followed by the lung cancer group with 48%. The extent of the cancerous growth did not appear to influence the incidence of positive results since 27 (32%) of the 85 patients with localized cancers had positive results, 43 (49%) of the 88 with regional spread had positive results and 26 (38%) of the 68 with distant metastases, had positive results.

Deletion from the data of non-cancerous disease patients with allergy, arthritis, acute tuberculosis or syphilis, temperature over 99.6°F and those with trauma, surgery or blood transfusion in the 10 days prior to the test, decreased the incidence of positive results in both groups by approximately the same extent, *i.e.*, 6%, thereby throwing additional doubt on the cancer specificity of the reaction.

Conclusion. (1) The Penn-Hall serofloccculation test was not found to be a satisfactory cancer selective procedure. Although it was seldom positive in normal individuals (5% of 129), it was positive in only 43% (96 of 241) of cancerous patients. Incidence of false positive results in 1608 non-cancerous patients was 19%. If patients with allergy, arthritis, acute tuberculosis or syphilis, temperature over 99.6°F and those with trauma, surgery or blood transfusion in prior 10 days were omitted as Penn and Hall suggested, the false positive results and the correct positive results were equally reduced by about 6%. (2) Positive results were more frequent among patients who had elevated blood sedimentation rates or who had inflammatory disease processes. As we evaluated, it is highly improbable that the reaction is cancer specific, a finding agreeing with the results of other investigators(3,4,5,6).

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Artifacts in Mast Cell Metachromasia Produced by Hyaluronidase Preparations.* (23795)

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In the course of recent experiments it was observed that, under specific conditions of staining with toluidine blue, mast cells of the hamster were rendered non-metachromatic by previous treatment with commercially prepared bovine testicular hyaluronidase. This finding is in direct contrast to that of other workers(3,5,15,17,18). Through a series of investigations it was possible to show that the observed effect was not the result of hyaluronidase activity but was instead an artifact.

Procedure. The tissue of the hamster studied most extensively was the cheek pouch, both in its normal state and when undergoing hyperplasia and neoplasia in response to applications of 9, 10-dimethyl-1,2-benzanthracene. Observations were also made on skin, mesentery, tongue and uterus. Tissues were fixed in Bouin's fluid, absolute ethyl alcohol and formalin, after which paraffin sections were prepared. Serial sections of each tissue were incubated 12 hours in hyaluronidase so-

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lutions§ containing 25 T.R.U./ml prepared both in distilled water and 0.12 M sodium chloride while the adjacent control section was exposed to same temperature and same solution without hyaluronidase. These tissues, along with a freshly hydrated section, were stained after the method of Moore and Schoenberg(14) for 20 minutes in 1 x 10^{-4} M solution of recrystallized toluidine blue, prepared in acetate and phosphate buffers of ionic strength 0.0025. Buffers were prepared for each pH unit from 2 to 7 inclusive according to the method of Miller and Golder(13). Tissue sections were equilibrated in buffer alone for 2 hours prior to staining. After staining, sections were washed in buffer and read while wet. Hyaluronidase solutions inactivated by heat were also used and as a check on effectiveness of heat as an enzyme inactivator, turbidimetric assay for hyaluronidase activity was carried out according to the method of Tolksdorf *et al.*(16). After incubation in sodium chloride solution of the enzyme, tissues were washed in sodium chloride solution followed by distilled water. Tissue sections exposed to aqueous solution of hyaluronidase were routinely washed in distilled water alone. On one occasion the latter were washed in 0.12 M sodium chloride and on another they were exposed 30 minutes to 3 changes of phosphate buffer solution at pH of 7.5 and ionic strength of 0.5.

Observations. Mast cells stained metachromatically at all pH's used. At pH 2 and 3, pretreatment of tissues with hyaluronidase in distilled water abolished all staining of mast cells irrespective of method of fixation.

At pH 4, the mast cells were metachromatic in hyaluronidase treated tissues but appeared distinctly lighter than control slides stained at same pH. There appeared to be no effect of exposure of tissues to water and elevated temperature alone. At pH 5, 6 and 7, exposure to a hyaluronidase solution had no effect upon metachromatic staining properties of the mast cells.

§ A comparison was made of the activity of 3 commercially available hyaluronidase preparations obtained from Nutritional Biochemicals Corp., Cleveland, O., Worthington Biochemical Corp., Freehold, N. J., Wyeth Laboratories, Philadelphia, Pa.

In contrast to above findings, tissues incubated in hyaluronidase solution prepared in 0.12 M sodium chloride and washed in sodium chloride after incubation, exhibited metachromatic staining of mast cells at pH 2 and 3. However, tissues incubated in distilled water preparation of hyaluronidase and then washed in 0.12 M sodium chloride revealed no staining of mast cells at these low pH values. If instead of 0.12 M sodium chloride, tissues were washed in phosphate buffer at pH 7.5 and ionic strength of 0.5, the mast cells stained metachromatic upon subsequent exposure to toluidine blue at pH 2 and 3.

Heating the aqueous enzyme solution 20 minutes in 60°C water bath did not alter its ability to prevent metachromasia of mast cells at pH 2 and 3 even though turbidimetric assay of the heated solution revealed an absence of hyaluronidase activity. However, when sodium chloride was added to a heated aqueous solution of hyaluronidase the mast cells exhibited metachromatic staining at pH 2 and 3.

The aqueous hyaluronidase solution which was subjected to a boiling water bath for 60 minutes became slightly opalescent. The mast cells of tissues incubated in this solution did stain when exposed to toluidine blue at pH 3 but staining was not as intense as that exhibited by mast cells subjected to sodium chloride hyaluronidase preparation.

Discussion. The experiments demonstrate that hyaluronidase activity *per se* was not responsible for loss of metachromatic staining of mast cells when exposure to heated aqueous enzyme preparations prevented all staining at pH 2 and 3 even though turbidimetric assay of the solutions revealed no active enzyme to be present. The fact that sodium chloride at the concentration used does not inhibit but rather enhances the activity of hyaluronidase (11) lends further support to the conclusion that loss of metachromatic staining of mast cells is not due to hyaluronidase activity *per se*. It appeared that some factor other than hyaluronidase activity, was responsible for the observed effect on mast cell staining.

Chauncey *et al.*(2) and Davidson and coworkers(4) have demonstrated numerous enzymes present as impurities in testicular hyal-

uronidase preparations. The possibility of some unknown "mucopolysaccharidase" in the preparations seems unlikely since sodium chloride was capable of abolishing the effect of the aqueous solution. Thus evidence points to the fact that it is some activity other than an enzymatic one which is responsible for rendering mast cells unstainable at low pH's.

It is known that anionic polyelectrolytes can combine with proteins at pH's sufficiently low to charge basic protein groups. If an anionic polyelectrolyte is metachromatic, such a combination may prevent its metachromatic staining. In fact, French and Benditt(6) and Kelly(9) have shown *in vitro* that basic proteins can suppress metachromasia and they do this more effectively at lower pH values. It is suggested that a masking of mast cell mucopolysaccharides by proteins present in the hyaluronidase preparations has occurred in the test system discussed here, creating an artifact which resembles enzymatic hydrolysis of the mucopolysaccharides. It is possible that hyaluronidase itself may be one of the masking proteins. There is evidence indicating that heparin is a competitive inhibitor of hyaluronidase(1,8,12). It forms with the enzyme salt-like bonds, which can be broken by increasing the ionic strength of the solvent (8). Since mast cells are thought to contain heparin(7,10), it may be postulated that the enzyme and heparin of mast cells formed a complex which prevented metachromatic staining of these cells. The pH dependency of the artifact and its failure to occur when a salt solution of hyaluronidase was used are consistent with this view. It is also possible that the masking effect may be due to basic nonprotein substances present in the hyaluronidase solution. The observation that mast cells could be stained at pH 3 if they were exposed for 30 minutes to a phosphate buffer solution at pH 7.5 and ionic strength of 0.5 after treatment with aqueous hyaluronidase, further suggests that some substances were removed by the relatively strong buffer and relatively high pH.

In the present day histochemical use of hyaluronidase preparations in particular and enzyme preparations in general, the observations presented here emphasize the need for cautious interpretation. The practice of running inactivated enzyme control sections is to be encouraged. Hyaluronidase prepared in salt solution is preferred over distilled water solutions of the enzyme.

Summary. 1) Commercially available hyaluronidase preparations appear to contain substances which are capable of preventing metachromatic staining of mast cells at pH 2 and 3 thus simulating enzymatic hydrolysis of stainable mucopolysaccharides. 2) Evidence suggests that staining is prevented by substances, perhaps proteins, which form a complex with mast cell mucopolysaccharides and prevent staining. It is possible that hyaluronidase itself may be one of the masking proteins. 3) The value of an inactivated enzyme control and use of salt solutions of hyaluronidase in histochemical studies is emphasized.

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Preparation of ECHO Complement-Fixing Antigens in Monkey Kidney Tissue Culture and Their Purification by Fluorocarbon. (23796)

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Increasing number of newly recognized ECHO viruses made it desirable to find a simple technic, such as the complement fixation (CF) test, for serological studies of these viruses, for determining antigenic relationships between types, and for typing newly isolated strains. In initial attempts to use CF antigens prepared in monkey kidney tissue culture in tests with ECHO antisera prepared in guinea pigs, difficulties were encountered in obtaining antigens with suitable titers which reacted specifically.

The present report describes the effect of fluorocarbon purification on ECHO complement-fixing antigens prepared in monkey kidney culture, the effect of maintenance media on the ECHO complement-fixing antigen yield, and the results obtained in CF tests using purified antigens from ECHO prototype strains and ECHO antisera prepared in guinea pigs and in monkeys.

Materials and methods. Viruses. The prototype strains of ECHO viruses passed in monkey kidney culture were furnished by Drs. J. L. Melnick, William McD. Hammon, and A. B. Sabin. *Tissue cultures.* Monkey kidney cultures were obtained from Microbiological Associates, Bethesda, Md. The cultures were grown for 6-8 days in a medium containing 2% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Hanks' solution. At the time of inoculation, the cultures in 32-oz. prescription bottles were washed twice with 40 ml of Hanks' solution. The maintenance media which were used during the study in bottle cultures appear in Table III. After comparison of CF antigen yield in various media, a medium containing 1% calf serum, 0.5% lactalbumin hydrolysate, and 98.5% Earle's solution was selected for routine use. The maintenance medium in tube cultures was Mixture 199(1). *Antisera. Guinea pig sera.* Guinea pigs were immunized with ECHO viruses grown in monkey kidney culture in Mixture 199. In some cases, crude tissue culture fluid, in others concentrated tissue culture material after treatment by sonic vibration, were used as immunization antigens. For anti-monkey kidney serum preparation, normal monkey kidney tissue culture cells in Mixture 199 were used. Detailed descriptions of these procedures and the immunization schedules will be published elsewhere. For CF tests, guinea pig sera were inactivated at 56°C for 30 minutes. *Monkey sera.* The lyophilized sera were obtained from the National Foundation for Infantile Paralysis.† The procedures used for preparation of these sera have been published by Archetti, Weston, and Wenner(2). Sera were reconstituted with distilled water and diluted 1:16 with buffered saline. The diluted sera were stored at −20°C. For CF tests, monkey sera were inactivated at 60°C for 20 minutes. Since the reconstituted monkey sera were anticomplementary, the lowest monkey serum dilution used was 1:32. *CF antigens.* Monkey kidney bottle cultures containing 40 ml of maintenance medium were inoculated with 0.3 ml of each of the prototype strains of ECHO types 1 to 14(3). The bottles were incubated at 37°C. After complete degeneration of tissue the bottles were stored at −20°C for 1-7 days. The majority of antigens were prepared as follows: 1) One part of fluorocarbon‡ was added into 2 parts of frozen and thawed tissue culture material. 2) The chilled mixture was homogenized in a VirTis Homogenizer at 20,000 rpm for 5 minutes. 3) The homogenate was centrifuged at 2,000 rpm for 10 minutes. 4) The aqueous phase contain-

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† The authors are indebted to Dr. A. B. Sabin for his assistance in obtaining these sera.

‡ Genetron 113, obtained from Allied Chemical & Dye Corp., New York City.

TABLE I. Effect of Fluorocarbon Treatment on Concentrated ECHO Type 12 CF Antigen. Homologous and heterologous CF reactions of ECHO guinea pig sera with untreated and fluorocarbon-treated E-12 antigens.

ECHO guinea pig antiserum	E-12 antigen, no treatment + (1:2) — Serum dilutions							E-12 antigen, treated by fluorocarbon (1:2) — Serum dilutions						
	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 1:2048	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 1:2048
E – 4	4	4	4	4	4	4		0	0	0	0	0	0	
5	4	4	4	4	3	0		0	0	0	0	0	0	
6	4	4	4	3	0	0		0	0	0	0	0	0	
12	4	4	4	4	4	4	2 0	4	4	4	4	4	4	2 1
14	4	4	4	4	4	4		0	0	0	0	0	0	

4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

ing the purified antigen was collected and stored at –20°C in screw-cap vials. In the early experiments when the maintenance medium in bottles was Mixture 199 without serum, and the virus yield was relatively low, concentration of antigens was necessary. The thawed tissue culture material was centrifuged in a Spinco ultracentrifuge at 10,000 rpm for 30 minutes, in Rotor #30. One part of fluorocarbon was added to 9 parts of supernatant, and the mixture was homogenized as described above. The aqueous phase was collected, 0.6% gelatin was added, and the material was concentrated by centrifugation in the Spinco at 30,000 rpm for 3 hours. The gelatin was added in order to keep the pellet compact, which made it possible to remove the supernatant without losing the pellet.§ The pellet was resuspended to 10X concentration in Mixture 199. All CF antigens were used unheated. *CF test.* The Bengtson complement fixation technic(4) with some modifications was used. Each new lot of commercial glycerinated hemolysin was titrated against 1:40 dilution of complement. Commercial lyophilized guinea pig complement was restored each Monday and titrated with a new lot of sheep cells. The same lot of restored complement and the same lot of cells were used in the tests of 5 following days. The restored complement was stored at 4°C. For the test 0.2 ml each of serum dilution, antigen, and complement (2 units) were mixed and incubated overnight at 4°C. Sensitized cells (0.4 ml) were added to give a final volume of 1.0 ml. The hemolysin and 2% sheep cells were mixed 10 minutes previously, during which time the test was removed from the refrigerator and held at room temperature. The tubes were incubated at 37°C for 1 hour, then held overnight at 4°C. The test was read the following morning. The titer was the highest dilution of serum or antigen giving 3 or 4+ fixation. A control titration of complement was included in each test, as well as the cell control and the anticomplementary controls of the lowest dilutions of antigens and sera.

Results. Effect of fluorocarbon treatment on ECHO antigens. Initially, fluorocarbon purifications were made with concentrated an-

§ This technic was suggested to us by Dr. S. Baron.

tigens prepared in Mixture 199 without serum, using 1 part of fluorocarbon in 9 parts of concentrated tissue culture material. This treatment removed sufficient non-specific reacting antigens that the purified antigens gave only specific fixation in CF tests when titrated against several ECHO guinea pig sera (Table I).

A more detailed study of the effect of fluorocarbon was made with unconcentrated ECHO antigens. It was found that 1 part of fluorocarbon in 2 parts of tissue culture material, homogenized at 20,000 rpm for 5 minutes, usually removed the reaction with host antigens (Table II). The effect, however, depended on the maintenance medium in the antigen. If maintenance medium was Mixture 199 without serum, one fluorocarbon treatment completely removed the host antigen reaction, whereas, if the maintenance medium was Mixture 199 + calf serum, many lots of antigens retained some host antigen reaction after one fluorocarbon treatment. In these cases a second fluorocarbon treatment was sufficient to abolish host antigen reaction. The purification was slightly improved if the cells were centrifuged down from antigens before fluorocarbon treatment, but in order to keep the purification procedure as simple as possible, and because even in the presence of cells, one or 2 fluorocarbon treatments removed the host antigen reaction completely, the precentrifugation of tissue culture materials was not adopted as standard procedure.

The effect of fluorocarbon on reducing the specific reaction of ECHO CF antigens seemed also to depend on the maintenance medium. Table II shows the effect of one and 2 treatments with fluorocarbon on ECHO type 4 antigens prepared in Mixture 199 and in Mixture 199 + 2% calf serum. One treatment did not decrease the antigen titer significantly in either antigen, but after the second treatment the antigen in Mixture 199 without serum did not have any specific reaction; in contrast to the antigen in Mixture 199 + 2% calf serum, which had practically the same antigen titer as after the first treatment. In similar experiments with other ECHO strains in Mixture 199 without serum, the decrease in the specific antigen titer after one fluorocarbon treatment was 2-fold or more. In the antigens containing serum, the decrease in the antigen titer after one or even 2 fluorocarbon treatments was less than 2-

TABLE II. Effect of Fluorocarbon Treatment on ECHO Type 4 CF Antigen in Mixture 199 and in Mixture 199 + 2% Calf Serum. Antigens titrated in CF test against type 4 monkey serum and anti-monkey kidney serum prepared in guinea pigs.

Antigen	Treatment of antigen	E-4 monkey serum dil. 1:128 — Antigen dilutions 1:1	1:2	1:4	1:8	Anti-monkey kidney guinea pig serum dil. 1:16 — Antigen dilutions 1:1	1:2	1:4	AC*	Infectivity titer (TCD_{50}/ml)
E-4 in Mixture 199, unconc.	F & T†	4	1	1	0	4	4	4	0	5.0
	F & T; homogenized	3	2	0	0	4	4	3	0	5.5
	F & T; 33% fluorocarbon ×1	3	2	0	0	0	0	0	0	5.0
	F & T; 33% fluorocarbon ×2	0	0	0	0	0	0	0	0	2.5
E-4 in Mixture 199 + 2% calf serum, unconc.	F & T	4	4	3	0	4	4	3	0	5.0
	F & T; homogenized	4	4	3	0	4	4	4	0	5.0
	F & T; 33% fluorocarbon ×1	4	4	2	0	0	0	0	0	4.5
	F & T; 33% fluorocarbon ×2	4	4	1	0	0	0	0	0	4.5

4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

* AC = anticomplementary reactions of antigens. † F & T = frozen and thawed.

TABLE III. Homologous and Heterologous CF Titers of ECHO Type 4, 5, 6, 11, 12, 13, and 14 Guinea Pig Sera.

Fluorocarbon treated ECHO CF antigens	ECHO guinea pig sera						
	E–4	E–5	E–6	E–11	E–12	E–13	E–14
E – 4	1024	0	0	0	0	0	0
5	0	4096	0	0	0	0	0
6	0	0	4096	0	0	0	0
11	0	0	0	2048	0	0	0
12	0	0	0	0	128	0	0
13	0	0	0	0	0*	1024	0
14	0	0	0	0	0	0	512
Homologous neutralization titer against 100 TCD_{50}	512	8000	64,000	8000	2048	512	2048

* $<1:2$ serum dilution. In all other instances, $0 = <1:8$ serum dilution.

fold.

Effect of maintenance medium on ECHO CF antigen titers. ECHO Type 4 antigen prepared in Mixture 199 had a titer of 1:1 before fluorocarbon treatment, whereas antigen prepared in a replicate bottle with Mixture 199 plus 2% calf serum had a titer of 1:4 (Table II). Similarly, ECHO Type 6 antigens prepared in replicate bottles with different media indicated that small amounts of calf serum increased both infectivity titers and antigen yields. Lactalbumin hydrolysate (0.5% to 1.0%) also increased antigen yields; however, the addition of cysteine (0.01%) and glucose (0.25%) to lactalbumin hydrolysate and calf serum had no apparent additional effect on antigen titers.

Medium containing 98.5% Earle's solution, 0.5% lactalbumin hydrolysate, and 1% calf serum was finally selected as the most desirable medium, since the use of Earle's solution eliminated the necessity (often encountered with Mixture 199 and Hanks' solution) for adjusting pH levels during the growth period. However, Earle's solution appeared to offer no other obvious advantages.

CF titers of ECHO antisera prepared in guinea pigs and monkeys. The homologous titers of guinea pig sera ranged between 1:128 and 1:4096 (Table III). The heterologous titers were all less than 1:8. No cross reaction (less than 1:2) could be demonstrated in repeated tests between type 13 antigen and type 12 guinea pig serum. The fluorocarbon purified antigens were also used in CF tests with monkey sera. However, the untreated antigens gave nonspecific reactions only with low dilutions (1:32 or less) of some sera.

The homologous and heterologous titers of monkey sera are shown in Table IV. The

TABLE IV. Homologous and Heterologous CF Titers of ECHO Monkey Sera.

ECHO CF antigen	ECHO monkey sera													
	E–1	E–2	E–3	E–4	E–5	E–6	E–7	E–8	E–9	E–10	E–11	E–12	E–13	E–14
E – 1	4096	0	0	0	0	0	0	32	0	0	32	32	512	0
2	0	1024	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1024	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	1024	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	4096	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	4096	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	2048	0	0	0	0	0	0	0
8	512	0	0	0	0	0	0	8192	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	1024	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	2048	0	0	0	0
11	32	0	0	0	0	0	0	0	0	0	4096	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	512	0	0
13	8192	0	0	0	0	0	0	512	0	0	0	512	8192	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	2048

$0 = <1:32$ serum dilution.

homologous titers ranged between 1:512 and 1:8192. Two-way cross reactions occurred mainly between types 1, 8, and 13. Type 13 antigen had high titer (1:512) one-way cross reaction with type 12 antiserum.

Discussion. Human and monkey tissue cultures are at present the only source of certain viruses for experimental studies; hence, tissue culture grown virus preparations must be used for CF antigens as well as for antiserum production in laboratory animals. Since animals homologous with the type of tissue culture used in the propagation of viruses (*e.g.* monkeys) are not always available, and since epithelial human and monkey tissue cultures apparently have common antigenic properties(5), difficulties will be encountered in CF tests in obtaining only specific fixation between virus antigen and antibody without fixation between tissue culture host antigens and antibodies. The purification of virus preparations with fluorocarbon reported by Gessler *et al.*(6) and application of the technic to tissue culture materials reported by Manson *et al.*(7) and by Hummeler and Hamparian(8), may solve these tissue culture host antigen problems in some cases. In the present study, fluorocarbon treatment proved a useful method for removing host antigen reaction in CF tests from ECHO antigens prepared in monkey kidney cultures. The effect of fluorocarbon on host antigens and also on specific ECHO virus antigens seems to depend largely on the amount of serum in tissue culture medium. In the present study, 2 fluorocarbon treatments (1 part of fluorocarbon to 2 parts of tissue culture material) decreased by 2.5 logs the infectivity titer of ECHO type 4 antigen in Mixture 199 without serum. Manson *et al.*(7) using 10% serum in medium, were able to treat HeLa grown poliovirus 3 times with fluorocarbon (9 parts of fluorocarbon in 1 part of tissue culture materials) before they could demonstrate decrease in infectivity titer. A preliminary study varying the amount of fluorocarbon and the number of treatments, therefore, may be necessary when a new type of medium and presumably a new type of virus will be exposed to fluorocarbon purification.

With the objective of typing newly isolated ECHO strains by the CF test, the purification of every isolation with fluorocarbon, although a simple procedure, might not be convenient. A more practical use of fluorocarbon purification may be the purification of immunization antigens. Whether or not fluorocarbon-purified antigens, which do not give host antigen fixation in CF tests, would produce any host antibodies in repeated immunization of guinea pigs, and whether these antigens would still be sufficiently active to produce specific antibodies, is unknown.

It is interesting to note a lack of correlation between infectivity titers and CF antigen titers of different types of ECHO viruses. For instance, type 5 antigen had 4.5 logs higher infectivity titer than type 4 antigen, but type 5 had 4 times lower CF antigen titer. A similar lack of correlation between neutralizing and CF antibody titers in ECHO antisera prepared in guinea pigs was observed (Table III).

The homologous and heterologous CF titers of ECHO monkey sera are similar to the results reported by Archetti, Weston, and Wenner(2), using ECHO antigens prepared in HeLa cells with the same sera. Minor differences are mainly in some cross reactions with lower titers. As with Archetti, Weston, and Wenner, the main two-way cross reactions were encountered between types 1, 8, and 13. The highest one-way cross reaction in both studies was obtained with type 13 antigen and type 12 antiserum. Since this cross reaction could not be obtained with type 12 guinea pig serum, the significance of this cross reaction in type 12 monkey serum may be questionable.

Summary. The fluorocarbon purification technic was applied to purification of complement-fixing antigens prepared in monkey kidney cultures from ECHO prototype strains. The effect of serum in tissue culture medium on the effect of fluorocarbon was studied. Adding serum to synthetic tissue culture medium improved the titers of ECHO CF antigens. Lactalbumin hydrolysate media containing serum represented the best media of several tried for the preparation of ECHO CF antigens in monkey kidney culture. The untreated antigens, when tested with guinea

pig sera prepared by immunizing animals with monkey kidney grown virus, gave strong monkey kidney tissue reactions. However, fluorocarbon treatment removed the nonspecific tissue reaction and "purified" antigens gave specific reactions in CF tests with ECHO antisera prepared in guinea pigs and in monkeys.

The valuable assistance of Hernon Fox in preparing the antigens is gratefully acknowledged.

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Functional Adrenocortical Homotransplants in the Golden Hamster.* (23797)

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No previous reports of transplantation of adrenal cortex in the hamster have been found in the literature. In this laboratory we have had no success in autologous transplantation of the adrenal cortex in adult hamsters, although such procedures are routine in the rat(1). However, we have found that fetal hamster adrenal cortex transplanted homologously to the cheek pouch of the adult hamster will grow, differentiate and function. This paper describes the technic and presents evidence for the first known cases of successful adrenocortical transplantation in the hamster.

Materials and methods. Two female golden hamsters (Mesocricetus auratus) of 14 days pregnancy were obtained from the Boston University Mammalian Genetics and Breeding Laboratory, following their observed matings, one with a sire of albino phenotype, the other with a sire of golden phenotype. The golden hamsters used as hosts were procured from a local independent hamstery and were about 3 months old, weighing from 60 to 80 g. The pregnant female was anesthetized with Nembutal, and the gravid uterus removed *in toto* to Ringer's solution. One by one, each fetus was excised from uterus and membranes, and the adrenal glands (where recoverable) were dissected out and placed on sterile gauze moistened with Ringer's solution. While the dissections were being performed by one person, the other operator prepared the cheek pouches of the anesthetized hosts and did the transplantations according to the technic for tumors described by Lutz *et al.*(2). No more than 5 minutes elapsed between excision of a fetal adrenal gland and its transplantation into a cheek pouch. Instruments were sterilized by boiling. Host cheek pouches were swabbed with a dilute concentration of Zephiran chloride (Winthrop) in Ringer's solution prior to transplanting the fetal organs. Eight fetal adrenal glands were transplanted homologously to cheek pouches of 6 intact host hamsters (Table I). On the 22nd day after transplantation the hosts were bilaterally adrenalectomized by way of 2 small dorsolateral incisions, in a single operation under light Nembutal anesthesia. Over a period of 15 to 28 days (Table I) after adrenalectomy these animals received 6 subcutaneous doses of cortisone (Cortone acetate, Sharp &

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TABLE I. Homotransplantation of Fetal Adrenal Glands to Adult Hamster Cheek Pouches; Summary of Procedure, Survival of Hosts, and Growth of Transplants.

Host hamster No.	Sex	No. fetal adrenals transpl.	Days after transplantation: Host adrenalectomy	Cortisone, 1.5-2.5 mg per dose	Saline water	Transplant excised from living host	Death of host	Vol of transplant at excision from host, in mm^3
1	♂	1	22	22-50 (6 doses)	22-50	154	159	8.0
2	♂	1	"	22-37 (6 doses)	22-39	67	73	10.0
3*	♂	2	"	"	"		53	.35
4*	♂	1	"	"	"		52	.4
5	♀	2	"	"	22-50	78	82	7.0
6	♂	1	"	"	"	78	83	6.5

* Died at 14 and 13 days after cessation of post-operative therapy.

Dohme, 25 mg/cc saline suspension) totaling about 14 mg, and saline drinking water (0.5% sodium chloride, 0.5% sodium citrate in tap water). Following cessation of all post-operative therapy, each animal was allowed to live for a further period of time equalling at least 4 times the expected survival time (7 days maximum) of adrenalectomized and untreated hamsters without transplants(3): *i.e.*, for at least 28 days (barring death of the animal before that interval elapsed). The transplant was then excised from the cheek pouch (excision of transplants from the pouch without harm to the animal is done routinely with tumors in this laboratory), and the animal was returned to its cage to determine the length of survival without the adrenal transplant. At death the hamster was examined for other possible causes of death (*e.g.* acute infection, diarrhea) and to ascertain the completeness of the adrenalectomy. During the course of the experiment observations and measurements of the transplants were made *in vivo* in anesthetized, everted pouches at magnifications up to 45X using transillumination. All hamsters were maintained in individual cages with Purina Laboratory Chow and drinking water (either saline or ordinary tap water) *ad libitum*. For histological examination all transplants and control adult normal adrenal glands were fixed in Helly's fluid, embedded in paraffin, sectioned at 6 μ and stained with Harris' hematoxylin and eosin Y. Specimens of fetal adrenal glands taken at time of transplantation were stained similarly but were fixed in Bouin's fluid and sectioned at 8 μ.

Results. Table I summarizes the procedure, growth of transplant, and survival time of each of the 6 animals. Body weights dropped 6 to 16% during the first 14 days after adrenalectomy. An apparent return to good health in 4 of the 6 animals was indicated by a slow gain in weight in Hamster 1, normal gains in Hamsters 2, 5 and 6, and normal activity and alertness of these animals. Regular observations of the transplants revealed vascularization by the host within 4 to 6 days after transplantation and a slow increase in size of the transplants over the approximate volume of 0.12 mm^3 as measured at time of excision from the fetuses. (The mean volume of 4 adult male hamster adrenals was 15 mm^3, and that of 10 female adrenals was 9 mm^3.) Body weights of Hamsters 1, 2, 5 and 6 dropped 11 to 23% during the 4 to 6 days between excision of transplant and death. In Hamsters 3 and 4, marked losses in weight beyond levels reached 14 days after adrenalectomy started on the day when therapy was withdrawn. Volumes of these transplants, fixed at time of death, were much smaller than those of the transplants in surviving animals.

Post-mortem examination of each animal revealed in no instance any gross evidence of acute infection which might have caused death, and verified the completeness of adrenalectomy in all cases.

Additional evidence for the adrenocortical nature of the transplants was provided by histological examination of the tissues.

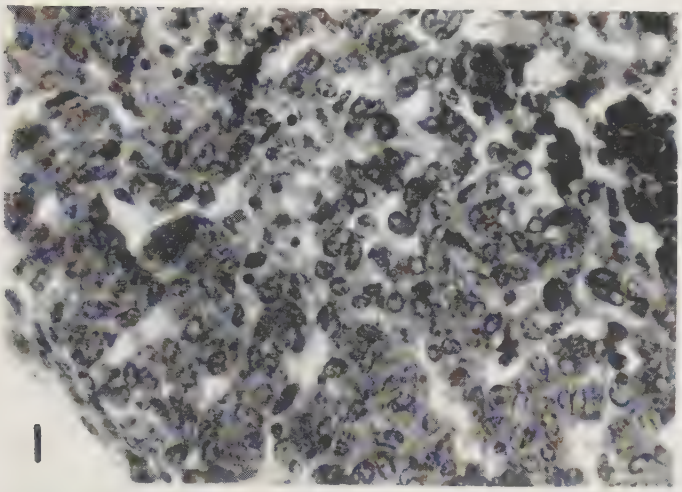

FIG. 1. 14-day fetal hamster adrenal × 430. Part of capsule at lower left. Note lack of cellular differentiation.

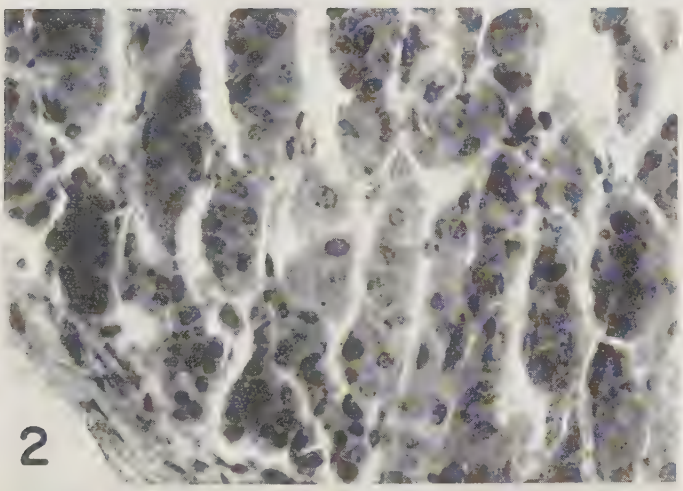

FIG. 2. Fetal adrenal homotransplant after 78 days in cheek pouch of Hamster #6 × 430. Part of capsule at lower left. Note zonation and cord-like arrangement of differentiated cortical cells.

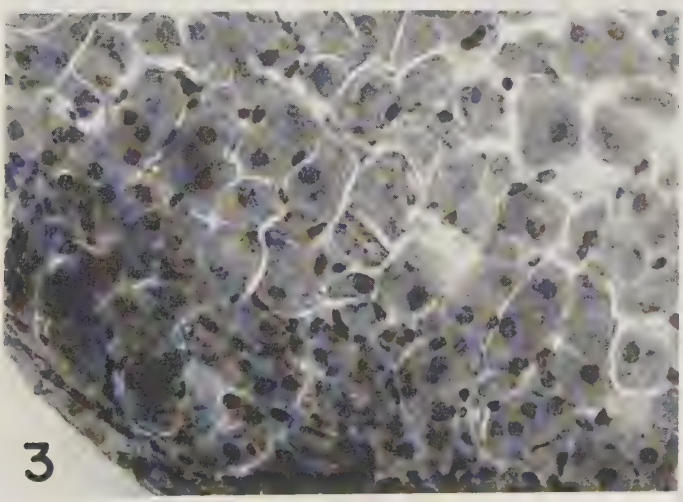

FIG. 3. Zona glomerulosa and part of zona fasciculata of adrenal cortex of adult female golden hamster × 430. Compare with Fig. 2.

Healthy cortical cells provided the main bulk of all transplants, including those of Hamsters 3 and 4. The cortical cells were arranged in small clusters or in curved cords of varying lengths. Two cell types were seen, one type having rather eosinophobic cytoplasm and believed to be cells of the zona glomerulosa, the other type being larger, having smoothly eosinophilic cytoplasm and believed to be cells of the zona fasciculata. In 3 transplants these cell types were segregated into zones and were arranged in fairly long cords (Fig. 2), somewhat similar to the arrangement in a typical adult adrenal (Fig. 3), with the glomerulosa cells lying in a narrow, compact layer directly under a definite capsule. (The adrenal cortex of the adult hamster has been well described in several recent papers including that of Meyers and Charipper(4)). No zona reticularis or medullary tissue was found, however, in the transplants. In the other 3 transplants the 2 cell types were arranged randomly and irregularly, and a discrete capsule was lacking. Small areas of dead or dying cortical cells and white blood cells were occasionally seen. A minor invasion by fibroblasts and connective tissue fibers from surrounding cheek pouch tissue or from the capsule occurred in most of the transplants. All transplants were well vascularized. No cellular connection was seen microscopically between the 2 original transplants in Hamster 5; the separate glands were equal in size and differentiation, and were bound together closely by a connective tissue sheath. The relation of the 2 glands in the transplant of Hamster 3 could not be determined, owing to the very small volume.

To explain the deaths of Hamsters 3 and 4 soon after cessation of postoperative therapy despite the presence of healthy adrenocortical cells in their transplants, we propose that the volume of functioning tissue was too small to maintain life.

All transplants showed marked cellular differentiation as well as increase in size when compared to the 14-day fetal adrenals fixed on the day of transplantation. The 14-day fetal adrenal (Fig. 1) is a very small, spherical mass of closely-packed, highly undifferentiated cells, recognizable as adrenal only by its unmistakable position in relation to the kidney. Cells of the transplants, although not always organized in radially-arranged rows or in segregated zones, were similar in appearance to those of an adult adrenal cortex.

Discussion. In a previous study in this laboratory, homotransplantation of fetal adrenals to 4 intact adult hamsters, with no subsequent adrenalectomy, resulted unexpectedly (in view of Halsted's principle) in clear histological evidence of growth and differentiation of transplants after 14 to 28 days in the cheek pouch, but provided no physiological evidence of their function; this result suggested the present study wherein adrenalectomy was delayed until 3 weeks after transplantation. Previously, homotransplantation of fetal adrenals to 2 adult hamsters on the day following adrenalectomy failed, although in the rat Martinovitch(5) has successfully homotransplanted infantile adrenal cortex to adrenalectomized hosts. Autotransplantation of half-glands at the time of complete adrenalectomy failed previously in 3 adult hamsters, the animals dying within a month despite post-operative therapy, the transplants showing mostly dead cortical cells. Auto-transplantation of partial glands at the time of incomplete (unintentional) adrenalectomy again resulted in complete failure of transplants to regenerate in 3 hamsters, although host survival was increased.

Successful autologous and homologous transplantation of adult adrenal cortex in the hamster may depend upon further studies of delayed or partial adrenalectomy and upon revision of the type and schedule of post-operative therapy.

Fetal hearts have also been successfully homotransplanted to the hamster cheek pouch (6), visible evidence for functional survival of cardiac tissue being regular contractions observed in the transplants up to 10 months after transplantation.

Summary. 1. Adrenal glands from golden hamster fetuses, transplanted homologously into cheek pouches of adult hamsters which were adrenalectomized 22 days after transplantation, maintained the untreated hosts for at least 4 times the expected survival time of adrenalectomized and untreated hamsters

without transplants. 2. When the adrenal transplants were excised from surviving hosts, after 67 to 154 days in the cheek pouch, the hosts died within 6 days. 3. Microscopic appearance of healthy cortical cells with some indication of adult arrangement in zones and cords, supported the physiological evidence for growth, differentiation and function of the adrenal transplants. This is the first known instance of successful adrenocortical transplantation in the hamster.

Acknowledgement is made to Maria Ramirez and Helen Melaragno for technical assistance, and to Frederick W. Maynard for photomicrographs.

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Human Parotid Gland Secretion: Flow Rate and Interrelationships of pH and Inorganic Components.*† (23798)

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Limited studies by Hildes and Ferguson (1) and Thaysen, Thorn, and Swartz(2) have indicated that pH, sodium, bicarbonate, and chloride levels of parotid saliva are directly related to rate of secretion, in contrast to potassium and phosphorus concentrations which were independent of flow-rate. The secretory rate is influenced by numerous local and systemic factors which include: method of stimulation, diet, sleep, dehydration, infectious diseases, emotional factors, psychological abnormalities, nervous disorders, and presence of various drugs. Thus any attempt to comprehend metabolic processes and secretion-mechanism of the salivary glands demands that the effect of all possible affecting variables be predetermined and controlled.

In a previous study(3), analysis of parotid saliva from 50 individuals permitted a preliminary establishment of normal mean and dispersion values for sodium, potassium, calcium, bicarbonate, chloride, phosphorus, and pH. The present publication reports the effect of various stimuli on secretory rate of the parotid gland and the interrelationships between rate of secretion and salivary composition.

Materials and methods. Parotid saliva was collected using a vacuum cup(4). Graduated collection tubes were immersed in iced water and mean flow-rate (ml/min) calculated by recording the time required for secretion of a fixed volume. The saliva was analyzed for sodium, potassium, calcium, chloride, and phosphorus. Samples for determination of pH and bicarbonate were collected under paraffin oil. Paraffin (masticatory), lemon flavor‡ (gustatory), and flavored chicle§ (masticatory and gustatory) were used to study effect of different stimulatory agents. *Analytical procedures*: Sodium and potassium values were determined with an internal standard (Li) flame photometer; calcium, by the Clark-Collip(5) modification of the Kramer-Tisdall principle; chloride, using Benotti's(6) modification of the Van Slyke prin-

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‡ Lemon Life Savers were used without any sucking or masticatory action.

§ Dentyne Gum.

TABLE I. Comparative Effect of Different Stimuli on Rate of Parotid Gland Secretion.*

Stimulus	1st sample	2nd sample	3rd sample	Avg rate, 3 samples
	ml/min.			
Paraffin	.58	.54	.62	.58
Lemon flavor†	.55	.64	.93	.71
Flavored chicle‡ (bolus renewed)	.72	.82	.79	.78

* Above data based on mean secretion rates from 5 subjects.

† Lemon Life Savers were used without any sucking or masticatory action.

‡ Dentyne gum.

ciple; bicarbonate, by titration method of Van Slyke(7); and phosphorus, by modification(3) of the King procedure. pH was measured in a Model G Beckman pH Meter.

Results. Effect of various oral stimuli: When a single bolus of gum§ (4.33 g) was chewed for a sustained period (during collection of 3 successive 5 ml saliva samples), secretion rate of successive samples was significantly decreased. When the bolus was discarded and a new portion of gum introduced every 5 minutes, flow rate of 3 successive 5 ml samples was more consistent. Similar results were obtained when "sweetened chicle" which contained sugar but no flavoring agent was employed. Examination of mean flow rate of 3 successive 12.5 ml saliva samples (Table I) showed that paraffin and flavored chicle (bolus renewed) evoked fairly consistent rates of secretion. Lemon flavor produced a highly variable inter-sample flow rate. Thus, if the inherent variability in flavored chicle was controlled by subjects chewing a fresh bolus every 5 minutes, the chicle was capable of producing a greater effect than either paraffin or lemon flavor.

Comparison of secretion from both parotid glands: No differences were evidenced in secretion rate and composition of saliva samples collected before and after breakfast (5 subjects). Above measurements were made on saliva from the right gland only. Three persons were used for comparison of flow rate and composition of saliva from both parotid glands. Samples were collected simultaneously from both glands using flavored chicle and alternating the side of mastication. The analysis (Table II) showed only slight differences in flow rate, pH, potassium, calcium, bicarbonate, and phosphorus titers of right and left glands. In contrast, a marked difference was observed in sodium and chloride levels of the 2 glands of certain subjects.

Effect of methacholine: Two subjects were given intramuscular injections of methacholine (10 mg). Chicle stimulated saliva samples were collected before and after administration of the drug (Table III). There was a marked increase in flow rate, chloride, sodium, and calcium levels. The latter 2 components were several times greater than the mean value found with 50 "normal" subjects and were greater than the maximum of "normal" range(3).

Secretion rate and salivary constituents: Parotid saliva samples were secured from 50 fasting subjects (before breakfast) using flavored chicle as stimulatory agent. Twenty-five ml of saliva (two 12.5 ml samples) were collected from each individual for 2 successive days. Samples A_1 and A_2 were collected on first day, while B_1 and B_2 were obtained the second day. Paired data analysis of the difference between individual mean flow rates of 4 specimens indicated that fraction A_1 had a significantly lower rate than the other 3 fractions. However, there was no

TABLE II. Comparison of Composition and Secretion Rate of Both Parotid Glands of 3 Subjects.

	Flow rate, ml/min.	pH	Na	K	Ca	Total cations	HCO_3	Cl	P	Total anions
			meq/l							
Right gland	.58	7.29	20.9	22.3	1.0	44.2	15.7	24.0	4.4	44.1
Left "	.58	7.28	19.1	22.8	1.0	42.9	15.4	20.2	4.1	39.7
Right gland	.75	7.40	41.8	19.7	1.6	63.1	18.6	38.6	4.2	61.4
Left "	.73	7.46	23.5	18.7	1.5	43.7	19.3	20.0	4.9	44.2
Right gland	.83	7.42	31.8	18.2	1.7	51.7	20.0	29.9	4.0	53.9
Left "	.86	7.50	39.2	19.7	1.9	60.8	23.2	27.1	3.9	54.2

TABLE III. Effect of Methacholine on the Parotid Secretion.

	Patient	Flow rate, ml/min.	Na	K	Ca	Total cations	Chloride
			meq/l				
Before admin.	G	.23	3.51	19.7	1.3	24.5	8.2
	P	.33	13.01	20.7	1.4	35.2	42.0
After "	G	4.44	47.0	16.0	3.3	66.3	11.2
	P	1.33	58.3	21.8	3.8	83.9	61.9

significant difference between fractions A_2, B_1, and B_2. Phi coefficient analysis of the relationship between rate of secretion and composition indicated a positive correlation between flow rate and sodium (+ 0.50), calcium (+ 0.38), bicarbonate (+ 0.55), and pH (+ 0.52). Only the potassium titer exhibited a negative correlation (–0.30), with rate of secretion. A positive correlation was noted between pH and sodium (+ 0.56), calcium (+ 0.36), bicarbonate (+ 0.64), and chloride (+ 0.28) levels. Intercorrelations between various parotid saliva electrolytes were also observed (Table IV). The above stated correlations were all significant: having a probability (P) of less than 0.05.

Discussion. Preparations which contain both masticatory and gustatory stimuli are most effective for eliciting secretion of saliva. However, if the gustatory stimulus is sugar, the effect is transitory; diminishing as concentration of available gustatory agent decreases. On the other hand, if total stimulus is renewed so that an optimal level of the gustatory agent (sugar) is present, the initial degree of maximal stimulation can be maintained.

Samples of parotid saliva collected from the same subjects in a fasting and postprandial state (A.M.) showed no significant differences in secretion rate, pH, or electrolyte composition. It was disclosed that the composition of parotid saliva can be markedly different in secretion from the 2 glands of the same person as well as between individuals.

TABLE IV. Intercorrelation between Parotid Saliva Components.

Component	Phi correlation coef.
Bicarbonate *vs*	
Na	+.44
Ca	+.48
Chloride *vs*	
Na	+.41
K	–.29
Sodium *vs* K	–.37

Marked changes in salivary composition were noted after injection of methacholine. This demonstrates the necessity that type of stimulant be one which elicits normal responses without introducing artifacts which might occur when stimulatory agent also produces vasodilatation or intense stimulation of post-ganglionic nerve fibers.

Statistical analysis of flow rates of 4 samples of parotid saliva collected from 50 normal subjects for a 2-day period showed that, except for initial sample, the parotid gland secretory rate was quite consistent when stimulus of constant intensity was employed. The lower rate of secretion in the initial sample apparently was due to apprehension by test subjects who were not familiar with the collection procedure. This variation was not encountered when subjects were given a pretesting "acquaintance session"(8).

Among constituents and properties of saliva reputed to be affected by rate of secretion are sodium, bicarbonate, chloride, and the pH (1,2). Our results confirmed these observations for sodium, bicarbonate, and pH. In addition, a positive correlation was found between flow rate and calcium titer, while the potassium titer was inversely related to flow rate. Only chloride and phosphorus levels showed no significant correlation with secretory rate. Intercorrelations between certain parotid saliva components were also observed.

It can be postulated from our findings that these relationships are, in part, due to carbonic anhydrase in the parotid glands(9,10). Under the influence of this enzyme the carbon dioxide and water, produced by metabolic processes of cells, would be converted into

carbonic acid with subsequent ionization into a hydrogen ion and a bicarbonate ion. The assumption that hydrogen ions so formed are transferred to blood or extravascular fluid while sodium ions from blood are transferred to cells, to become associated with bicarbonate ions and be secreted in the saliva, would account for the rise in pH, sodium, and bicarbonate levels produced by increasing secretion rates.

Summary. Stimulated parotid saliva was collected from 50 individuals using flavored chicle as the stimulatory agent. Analysis of the flow rate and composition indicated a significant positive correlation between the flow rate and the pH, sodium, calcium, and bicarbonate contents. Intercorrelations between the various salivary constituents were also noted. No differences were found in the composition of saliva samples collected in the fasting and postprandial state. The composition of the parotid secretion was found to vary not only from person to person but between the two glands in the same person.

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Effect of Glucagon on Growth of Chick Embryo. (23799)

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It has been reported that glucagon causes a significant increase in the weight of Leghorn chick embryos and this finding has been used as evidence that glucagon is a growth-stimulating factor(1). In these experiments a single dose of glucagon (5 or 25 μg) was injected "in" the chorioallantoic membrane on the ninth day of incubation, and the embryos weighed 4, 7 or 9 days later. Since relatively crude preparations of glucagon* were used in these studies, it was felt that the experiments should be repeated using highly purified, crystalline glucagon.

Methods. Five experiments were done using a total of 113 control and 111 experimental White Leghorn x Delaware (Hyline strain) chick embryos, incubated at 37°C in a forced-draft incubator. The doses of crystalline glucagon† per embryo ranged from 5 to 80 μg administered in 1 to 5 injections over the course of 1 to 9 days. The injections were done according to the following technics: After puncturing the air space with a sharp needle, a hole was carefully drilled through the shell over the embryo. The shell membrane was moistened with sterile saline and carefully torn with a needle. After ascertaining that the chorioallantoic membrane had dropped, the tip of a hypodermic needle was inserted into the opening and the appropriate volume of solution delivered. The openings in the shell were sealed with paraffin, after

* Lot No. 208-108B-234: contains 20% of the activity of crystalline glucagon.

† Prepared by Dr. A. Staub according to the method of Staub *et al.*(3).

TABLE I. Effect of Glucagon on Chick Embryo Weight.

Exp.	No. of embryos	Dose of glucagon (μg)	Age of embryos (days) at Onset of exp.	Age of embryos (days) at End of exp.	Mean wt of embryos (g)	Diff. between control and exp. (g)	Significance of diff. (p)	Mortality, No. (%)
1	Cont. 12	0	9	½: 16 ½: 18*	16.54	+ .67	.54	0
	Exp. 11	5	9	" "	17.22			1 (8.3)
	Cont. 15	0	9	" "	16.50	+ .25	.82	1 (5.5)
	Exp. 16	10	9	" "	16.75			0
2	Cont. 19	0	9	19	18.63	+1.38	.07	2 (10)
	Exp. 21	10 × 4	9	19	20.01			0
3	Cont. 24	0	9	17	15.06	− .20	.70	0
	Exp. 24	20 × 4	9	17	14.86			0
4	Cont. 20	0	11	19	21.00	− .67	.30	4 (16.6)
	Exp. 24	5 × 5	11	19	20.33			0
5	Cont. 23	0	11	19	21.81	−1.17	.22	0
	Exp. 15	2.5 × 4	11	19	20.63			8 (34.7)

* In view of the small No. of cases in this exp., the means for 16- and 18-day embryos in each of the 2 groups (Cont. and Exp.) were averaged.

the injection, and the egg returned to the incubator. The glucagon solutions were made up freshly for each experiment using sterile Pannett-Compton's solution(2) after dissolving the glucagon in 2 drops of 0.2% NaOH. The glucagon concentration was adjusted so that each dose was administered in a volume of 0.2 ml. Control embryos were injected with 0.2 ml of the Pannett-Compton-NaOH solution. At the end of each experiment the embryos were removed from their shell and extra-embryonic membranes. They were then carefully dried with filter paper and weighed within 2 minutes to avoid weighing errors due to desiccation.

Results. Table I gives a summary of the data from the 5 experiments. It is apparent that highly purified, crystalline glucagon administered in a wide range of doses has no significant effect on chick embryo weight.

The present findings do not confirm the report of Cavallero that glucagon increases chick embryo weight(1). The experimental conditions used were similar to those used by Cavallero except for the difference in the purity of the glucagon used. Furthermore, there was no dose-response relationship in Cavallero's experiments, a 25 μg dose of glucagon did not elicit a greater response than that achieved with a 5 μg dose. These considerations suggest that Cavallero's findings may have been due to a contaminant(s) in his relatively impure glucagon preparation. It is of interest that one of us reported(4) a growth stimulating effect in the young, hypophysectomized rat with crude glucagon preparations and that this effect could not be confirmed by subsequent workers(5,6) who used much purer glucagon preparations.

By the last third of the incubation period the growth of the chick embryo is probably under the control of its own endocrine system. Inhibition of thyroid function by goitrogenic compounds(7,8,9) or removal of the developing hypophysis by "partial decapitation"(10, 11,12) results in failure to attain normal body weight.

Growth stimulation in the chick embryo has been reported from thyroxine(13) or pituitary growth hormone(14) administration. Insulin, although causing a hypoglycemic and a teratogenic effect when administered to the young embryo(15) does not alter body weight when given at later stages(1).

Summary. The effect of highly purified, crystalline glucagon on the weight of chick embryos was studied in a series of 5 experiments and using a wide range of doses. No significant effect on weight of embryos was observed. It is suggested that a previous report of significant increases in embryo weight induced by glucagon may have been due to contaminants in the relatively crude glucagon preparation used.

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Hypercholesteremia and Atherosclerosis Induced in Rabbits by Purified High Fat Rations Devoid of Cholesterol.* (23800)

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Investigations in humans(1-6) have recently established that a difference in cholesterol levels can be induced by dietary use of certain vegetable oils and some fish oils as compared to hard animal fats and hydrogenated fats. A large dietary intake of certain hard animal fats or hydrogenated fat is capable of elevating blood cholesterol levels. Conversely highly unsaturated vegetable oils either have no such effect or will actually lower plasma cholesterol levels. Speculation as to agents in fats or oils which affect cholesteremia has included: saturated or unsaturated fatty acids(1), essential or polyunsaturated fatty acids(7), beta-sitosterol(8) and short chain fatty acids(1). Following the classic studies of Anitschkow(9) numerous investigators have produced hypercholesteremia and aortic atheroma in rabbits by adding cholesterol to commercial rabbit feeds. Kritchevsky *et al.*(10) showed that type of fat was important in determining extent of this effect. They found that, in the presence of exogenous cholesterol, partially hydrogenated vegetable shortening produced higher blood cholesterol values and more severe aortic lesions than did corn oil. In the absence of dietary cholesterol, Kritchevsky found negligible atheroma production with either vegetable shortening (Primex) or corn oil when both were fed at 9% of the diet. This latter finding led Deuel and Rieser(11) to the conclusion that. "These data likewise refute the concept that fat *per se* has an atherogenic effect." Steiner and Dayton(12) more recently reported that blood cholesterol levels could be elevated 4-5 times in rabbits without dietary cholesterol by prolonged feeding of diets containing 50-75% ground peanuts. They also reported small areas of gross aortic atherosclerosis in 2 of 33 rabbits after 5 to 12 months, thus suggesting that atherosclerosis could be induced by diets low in cholesterol. Use of diets of known controlled chemical composition makes possible more accurate control of all dietary components than can be attained with the usual rabbit diet. In this paper we describe 2 rabbit experiments using the purified diet of Thacker(13).

Methods. Male New Zealand albino rabbits averaging 1.8 kg in weight were housed in individual cages. They were divided into groups of comparable weight. Diet consumption was estimated daily and body weight weekly. Rabbits were bled from the marginal ear vein before initial feeding and at suitable

* This work was reported in part to 132nd Nat. Meeting of Am. Chem. Soc., N. Y., Sept., 1957.

TABLE I. Composition of Purified Diets.

Ingredient	% composition
Alphacel*	10., 9.75, 9.25 or 8.
Casein†	25.
Dextrin	39.9
Cholesterol‡	.0, .25, .75 or 2.
Fat§	20.
Macro minerals	5.
Minor "	.1
	100.

* Nutritional Biochemicals Corp., Cleveland, O.

† Commercial grade: Borden Co., N. Y.

‡ Cholesterol levels varied at expense of Alphacel.

§ Hydrogenated shortening (Primex), hydrogenated coconut oil (Hydrol) or safflower oil.

Macro minerals, g — $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$ 308.2, $Ca(H_2PO_4)_2$ 104.7, K_2HPO_4 218.7, KCL 124.7, NaCl 77.0, $CaCO_3$ 68.5, $3\,MgCO_3 \cdot Mg(OH)_2 : 3H_2O$ 35.1, $MgSO_4$ 38.3.

Minor minerals, g—$FeC_6H_5O_7 \cdot 5H_2O$ 453.85, $CuSO_4 \cdot 5H_2O$ 28.15, $MnSO_4 \cdot H_2O$ 16.5, KI 1.5.

Vitamins added as follows: mg or I.U./100 g diet—B_1 HCl .07, B_2 .6, dl calcium pantothenate 1.5, B_6 HCl .7, niacin 20., choline chloride 100, betaine chloride 100, inositol 10, para-aminobenzoic acid .2, folic acid .1, d biotin .05, vit. A acetate 665 I.U., B_{12} .005, D_2 850 I.U., E 7.5 I.U., menadione .075.

intervals thereafter. Heparin was used as an anticoagulant. Plasma cholesterol was estimated by a modified method using the Abell (14) procedure for saponification and extraction except that 3 Skellysolve B extractions were used and the entire supernatant was removed after each extraction. After evaporation of combined supernatants to dryness under nitrogen at 60°C, the residue was taken up in chloroform to standard volume, filtered, and cholesterol content determined on aliquots by the method of Kenney(15) at 420 *mu* and color development at 37°C for 30 min. Aortae were stained with Sudan IV and graded for gross atherosclerotic lesions by 2 independent observers using a scale of 0-4 (ranging from none to severe). For grading purposes aortae were divided into 2 areas *i.e.* above intercostals, and intercostals and below. Dietary fat (Table I) consisted of partially hydrogenated vegetable shortening (Primex†), hydrogenated coconut oil (Hydrol‡) or safflower oil§ (containing about 70% linoleic acid). Fat in these diets accounts for approximately 40% of caloric value. When cholesterol was present it was mixed with other dry ingredients instead of being dissolved in fat. It is necessary to train rabbits to eat this purified diet by mixing it with 3% of alfalfa meal for the first week. *I. Cholesterol-containing diets*: Seventy-two rabbits were divided into 6 groups of 12 each and fed *ad lib*. Three groups were fed 20% Primex diet with cholesterol levels of 0.25, 0.75 and 2.0% respectively. Three other groups were fed 20% safflower oil diet with the same 3 levels of cholesterol. *II. Cholesterol-free diets*: Twenty rabbits were divided into 2 groups of 10 each. One group was fed 20% Hydrol diet and the other 20% safflower oil diet. All rabbits were pair-fed, that is daily food ingestion was limited to an amount which all rabbits would eat.

Results. I. Cholesterol-containing diets. Body weight, food consumption, mortality, and aortae atheroma data are presented in Table II. Animals on the 2% cholesterol diets were sacrificed and autopsied after 67 days, the 0.75% cholesterol group after 75 days, and the 0.25% cholesterol group after 103 days. No differences in the degree of lesions between survivors on various diets could be detected, however, mortality was most severe for all groups on hydrogenated shortening. At all 3 levels of dietary cholesterol rabbits on safflower oil gained weight more rapidly and ate more food than did rabbits on shortening.

In addition to poor growth and food consumption, many animals on hydrogenated shortening diet showed loss of hair, suggestive of essential fatty acid deficiency. Some rabbits on each diet developed a jaundiced condition. The cause of jaundice under these conditions is not understood but is perhaps related to hypercholesteremia commonly occurring with biliary obstruction.

Changes in average blood cholesterol values for the groups are shown in Fig. 1. Even though rabbits on hydrogenated shortening diet ate less food than their counterparts receiving safflower oil and thus ingested less cholesterol, their average plasma cholesterol levels were higher than those of safflower oil

† Procter and Gamble, Cincinnati, O.

‡ Durkee Famous Foods, Chicago, Ill. Iodine value approximately 4.

§ Pacific Vegetable Oil Corp., San Francisco, Calif.

TABLE II. Aortae Grading, Body Weight, Food Consumption, and Mortality of Rabbits Fed Cholesterol-Supplemented Diets.

Diet		Avg terminal aortae grading*	Avg terminal wt (kg)	Avg food consumption (g/rabbit/day)	% mortality†
Fat	Cholesterol (%)				
20% hydrogenated shortening	2.	.96‡	1.77	65	33
	.75	1.05‡	1.96	57	67
	.25	1.03‡	2.57	66	25
20% safflower oil	2.	1.19‡	2.65	84	8
	.75	1.07‡	2.78	71	33
	.25	1.19‡	3.22	87	0

* Mean of areas (above intercostals; intercostals and below).
† Initially each group contained 12 animals.
‡ Difference between hydrogenated shortening and safflower oil groups not statistically significant.

rabbits. This was true for all 3 dietary regimens.

Statistical analysis of differences between groups (by t test) was applicable only when plasma cholesterol determinations were done on individuals (after 45 days). Analysis of such data demonstrated that in most cases differences in plasma cholesterol levels between hydrogenated shortening and safflower oil groups were significant ($P<0.05$). One exception was found in groups on diets containing 0.25% cholesterol. At end of experimental period, plasma cholesterol levels were not significantly different between these 2 groups.

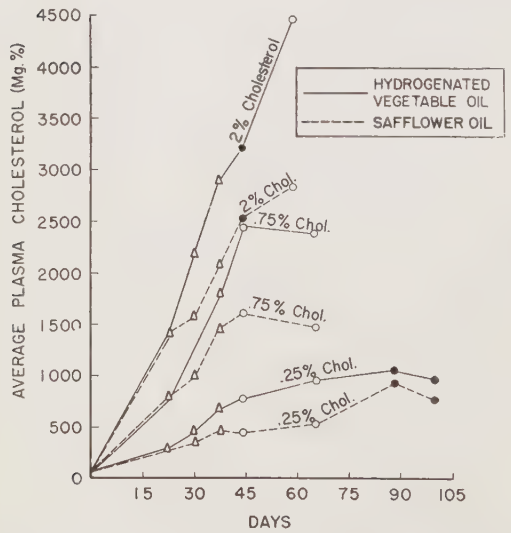

FIG. 1. Effect of exogenous cholesterol on plasma cholesterol levels (hydrogenated shortening *vs* safflower oil). △—Pooled plasma not subject to statistical analysis. ○—Statistically significant difference between groups at a given amount of exogenous cholesterol ($P<0.05$). ●—Differences between groups not statistically significant.

II. Cholesterol-free diets. Because the above experiment did not distinguish between types of fat as to effects on production of atheroma, it was thought that perhaps all 3 levels of exogenous cholesterol so overwhelmed the animal that atheroma were produced in presence of either type of fat. Accordingly, an experiment was designed to compare hydrogenated coconut oil, an almost completely saturated fat, against safflower oil in the absence of dietary cholesterol. Plasma cholesterol values for this experiment are shown in Table III. After 100 days on experimental diets, plasma cholesterol level was elevated 12 times over normal (0 days) by 20% hydrogenated coconut oil diet and only 1.5 times by 20% safflower oil diet. All animals on hydrogenated coconut oil surviving to termination of experiment had clear-cut aortic atheromatous lesions with average degree of severity of 1.4, whereas only 4 of 10 rabbits on safflower oil showed any aortic involvement, as manifested by slight sudanophilia. Difference in appearance between stained aortae from the 2 dietary groups was visually dramatic.

For histologic studies‖ aortae were fixed in neutral 10% formalin, sectioned and stained with hematoxylin-eosin, Schiff's periodic acid, Mallory's aniline blue connective tissue stain, Wilder's silver reticulum stain and Unna's orcein stain for elastic fibers. To be able to examine as much as possible of lesions present in the aorta the whole length of the aorta

‖ We are indebted to Dr. R. J. Stein for histological examination of the aortae.

TABLE III. Individual Plasma Cholesterol Levels and Degree of Atheroma of Rabbits on Cholesterol-Free Diets.

Diet	Plasma cholesterol (mg %)			Terminal aortae grading	
	0 days	52 days	100 days	Above intercostals	Intercostals and below
20% hydrogenated coconut oil	43	476	567	1.3	.6
	107	751	1062	1.9	1.7
	66	226	750	2.2	.4
	51	412	719	1.9	.9
	66	771	653	3.0	1.0
	68	459	dead		
	36	dead	"		
	59	555	729	1.7	1.0
	59	756	dead		
	69	386	627	.8	.9
Avg	62	532	730	1.9	.9
20% safflower oil	88	143	136	.0	.0
	43	117	101	.0	.05
	116	118	113	.0	.0
	106	143	141	.1	.1
	39	109	62	.0	.0
	lost	116	139	.0	.0
	125	213	156	.1	.0
	45	79	75	.05	.0
	45	119	86	.0	.0
	74	149	135	.0	.0
Avg	76	131	114	.03	.02

(arch, thoracic and abdominal aorta) was rolled upon itself like a deflated fire-hose, tied around its circumference with black surgical silk and sections taken through the whole. In this manner one was able to examine the various regions of the aorta in one slide (Fig. 2-a). Microscopically, the lesions exhibited marked intimal changes and to a lesser degree changes within the media. As shown in Fig. 2 (b,c,d) the intima was markedly thickened and almost entirely composed of oval and round foam-like cells. These cells contained nuclei that were round and some of which were vesicular with a scanty amount of chromatin material and others definitely pyknotic. Cytoplasm stained very lightly with eosin and exhibited a fibrillar-like architecture. Lining this intimal thickening a few normal appearing endothelial cells could be seen. The histology of this intimal change was characteristic of a true experimental atherosclerosis. Changes in the media were characterized by thickening and splitting of the elastic fibers with appearance of more than usual cellularity of the collagen between these elastic membranes.

Of the original 10 animals per group, 3 on hydrogenated coconut oil failed to survive the experimental period while all survived on safflower oil. Rabbits on the hydrogenated coconut oil diet evidenced considerable loss of hair and a general "ruffled" appearance (Fig. 3). Those on safflower oil were robust in appearance and would have consumed much larger quantities of diet if their intake had not been limited. Two animals on hydrogenated coconut oil developed jaundice while no jaundice occurred in the animals on safflower oil. The over-all average feed efficiency (food eaten/wt. gained) for rabbits on safflower oil was 3.92 (2.64-5.22) as compared to 5.78 (3.42-13.32) for rabbits on hydrogenated coconut oil.

Discussion. This study indicates that relative ability of different dietary fats to induce atheromatous lesions can be more clearly differentiated by cholesterol-free dietary procedure than by traditional cholesterol-feeding. This experimental technic obviates feeding unphysiologically high levels of exogenous cholesterol and reflects the cholesterolgenic property of the diet itself.

The difference in these experiments between safflower oil and hydrogenated coconut oil

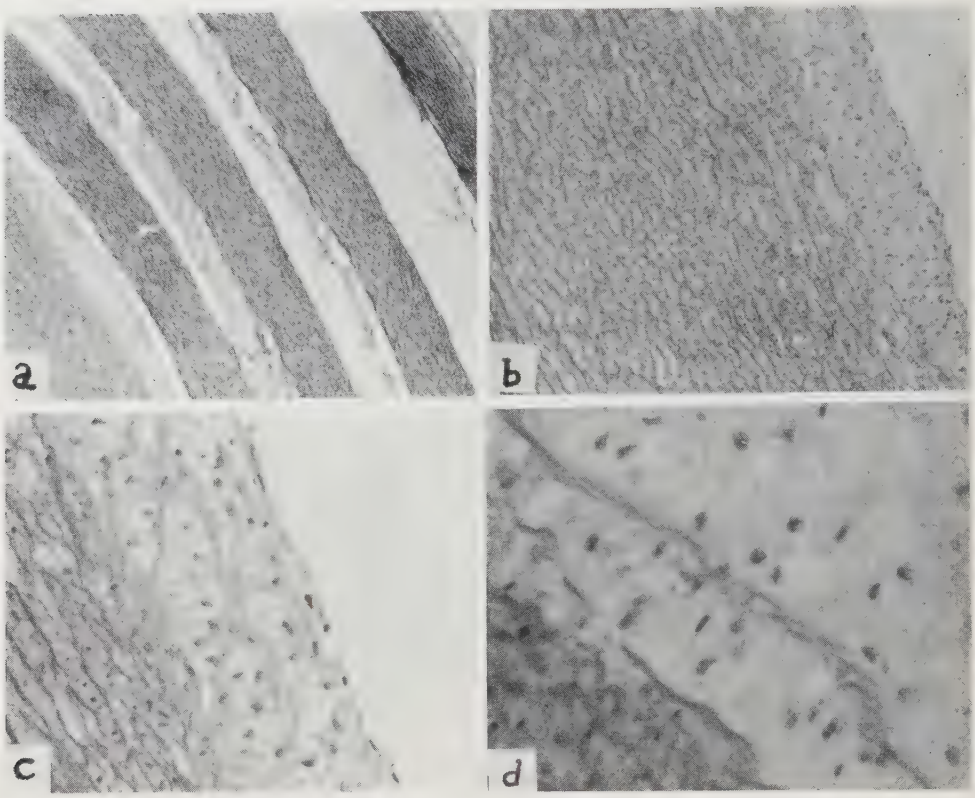

FIG. 2. Sections of aortae from rabbits fed cholesterol-free diets containing hydrogenated coconut oil.

might be due to (a) a hypercholesteremic effect of short chain saturated fatty acids and/or unnatural fatty acid isomers present in hydrogenated coconut oil (b) development of essential fatty acid deficiency in the coconut oil group (c) the relatively high amount of beta-sitosterol in safflower oil (0.40%)¶ as compared to hydrogenated coconut oil (0.13%)¶ (d) a hypercholesteremic effect of saturated fatty acids in coconut oil or a hypocholesteremic effect of unsaturated fatty acids in safflower oil (e) unknown substances in the fats, or (f) a combination of these possibilities. Two groups have reported that, in absence of dietary cholesterol, hydrogenated fat feeding did not produce atheroma in rabbits. Kritchevsky, *et al.*(10) found negligible atheroma production with either hydrogenated vegetable shortening (Primex) or corn oil when both were fed at 9% of the diet. Van Handel, *et al.*(16) reported that isocaloric substitution of hydrogenated vegetable fat (linoleate 1.5%) for liquid cottonseed or corn oil had no cholesteremic effect. Shortenings of the type used by Kritchevsky still contain up to 12% linoleic acid after hydrogenation(17) and differ considerably in this respect from hydrogenated coconut oil. Also both of these groups of investigators used a commercial rabbit diet and undoubtedly these preparations

¶ Total Lieberman-Burchard positive substances estimated using a beta-sitosterol standard.

FIG. 3. Appearance of rabbits after 12 wk on purified rations consisting of either 20% hydrogenated coconut oil or safflower oil.

contain appreciable amounts of unsaturated fat. The relatively high content of short chain fatty acids in coconut oil as compared to hydrogenated vegetable shortening or hydrogenated cottonseed is also apparent. Obviously the hydrogenated coconut oil as a sole source of fat leads to an aberrant cholesterol metabolism. Whether this is due to greater synthesis, to a failure in excretion, or other factors is open to further investigation.

Holman and Peifer(18) produced essential fatty acid deficiency in rabbits by feeding diets containing 2% hydrogenated coconut oil and 1% cholesterol. Using higher levels of hydrogenated fat we have produced a similar deficiency either with or without exogenous cholesterol. This latter finding concurs with the observation of Deuel, *et al.*(19) that development of an essential fatty acid deficiency can be hastened in the rat by addition of hydrogenated coconut oil to an otherwise fat-free diet.

Recently we found that the time period necessary to produce atheroma in rabbits fed hydrogenated coconut oil may vary. It appears likely that both blood cholesterol levels and time are important factors in determining extent of atheroma production.

Summary. Rabbits fed cholesterol-supplemented purified diets containing 20% of hydrogenated shortening or safflower oil showed lower plasma cholesterol levels on safflower oil diets, but only negligible differences between oils with respect to aortic atheroma production. Similar studies with cholesterol-free diets showed that rabbits on hydrogenated coconut oil are much more prone to hypercholesteremia than rabbits on safflower oil diets. In these latter studies aortic atheromatous lesions developed in all rabbits in 16 weeks on 20% of saturated fat as contrasted to negligible lesion production on 20% safflower oil.

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Resistance of *Salmonella typhimurium* to Tetracyclines. (23801)

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The problem of development of resistance by bacteria to antibiotic substances is so well known as to need no comment. As concerns development of resistance by enteric bacteria as a result of prophylactic and therapeutic use of antibiotics, the observations of LeMinor *et al.*(1), Buttiaux *et al.*(2), Smith(3,4) and Smith and Crabb(5,6) are pertinent. These workers observed the emergence of resistant strains of *Escherichia coli* in man and animals following prophylactic and therapeutic use of the agents. Further, Smith and Crabb(5) noted rapid emergence of tetracycline resistant strains of *E. coli* in fowls and swine maintained on feeds which contained these substances.

Materials and methods. Since the inclusion of antibiotic substances in feeds has become such a general practice it was thought desirable to examine the reaction of *Salmonella* cultures to these substances. *Salmonella typhimurium* is the serotype most frequently found in man and animals, and therefore, was chosen for test. Advantage was taken of a large collection of *Salmonella* cultures isolated prior to 1948, *i.e.*, before inclusion of antibiotic substances in animal feeds and before the broad spectrum antibiotics came into general therapeutic use. These cultures had been stored in infusion agar stabs in tubes stoppered with paraffined corks and had not been transferred since they were received and identified. They had been kept in the dark at room temperature for periods varying from 10 to 18 years. From these cultures were selected 100 strains of *S. typhimurium* isolated from animals and an equal number isolated from man. Of the animal strains, 90 were derived from fowls and 10 from swine. From cultures isolated subsequent to Jan. 1956, were selected 100 cultures of *S. typhimurium* isolated from fowls and 100 cultures isolated from man. In all instances an effort was made to select strains of wide geographic distribution, so that cultures from all sections of the country were included. The antibiotic substances tested were tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, dihydrostreptomycin, polymyxin B, penicillin, and erythromycin. In screening the cultures, blood agar base plates were streaked with individual cultures and antibiotic discs of intermediate concentration placed on the surface as soon as the inoculum was dry. Results were read after overnight incubation at 35°C. Early in the work it became apparent that significant results would not be obtained with penicillin, erythromycin, dihydrostreptomycin, and polymyxin B. All cultures tested with penicillin and erythromycin were resistant to the action of these substances and equal numbers of old and new isolates were resistant to the action of streptomycin. All cultures tested with polymyxin B gave a narrow clear zone of inhibition. With the exception of dihydrostreptomycin, which was used in examination of one-half of the cultures, the above substances were used in the examination of only a quarter of the strains. All cultures tested also were susceptible to the action of chloramphenicol, but since this substance has been employed so widely in the treatment of enteric infections, its use was continued throughout the work. After cultures had been screened with discs, all tetracycline resistant cultures and an equal number of susceptible strains were examined in tube dilution tests with chlortetracycline. Susceptible cultures were made up of equal numbers of old and new isolates. Tests were done in the usual manner with serial dilutions of the antibiotic which ranged in final concentrations from 0.10 μg to 200 μg/ml. To 0.5 ml of the antibiotic dilutions in distilled water were added 1.5 ml portions of 18-hour cultures which had been diluted 1 to 400 in trypticase soy broth. Results were read after overnight incubation.

Results. The only significant differences noted in reactions of cultures were obtained with tetracyclines. Among cultures isolated

prior to 1948, no resistant cultures were found. Among recently isolated strains, 9% from fowls and 5% from man appeared completely resistant as judged by their reactions toward the discs containing these substances. As would be expected, cultures which were resistant to one of the tetracyclines were resistant to all.

In every instance the results obtained by the disc method were confirmed. Among the susceptible strains, no differences were apparent between the old and new isolates. With the exception of one culture which produced visible growth in 6.25 μg/ml, the cultures which appeared susceptible by the disc method were inhibited by concentrations of chlortetracycline higher than 3.125 μg/ml. On the contrary, with one exception which grew only in 25 μg/ml, the resistant strains grew well in 50 μg concentration of the antibiotic and half grew in a concentration of 100 μg/ml.

From these results it is apparent that *Salmonella* cultures which are resistant to tetracyclines occur more frequently today than they did before these agents were used. Had a larger number of cultures isolated prior to 1948 been examined, some resistant cultures might have been found. However, the fact that no resistant cultures were found among 200 strains of *S. typhimurium* indicates that resistant cultures previously occurred very rarely. The fact that a significant increase in occurrence of resistant strains was found only in tetracyclines probably is a reflection of the widespread use of these substances in animal feeds. With their continued inclusion in feedstuffs and their continued therapeutic use, an increase in the proportion of resistant strains may be expected. The demonstration of resistance in twice as many cultures derived from fowls as in cultures derived from man also probably is related to the inclusion of tetracyclines in poultry feeds, and may be contrasted with the lack of resistance to chloramphenicol which is not so used.

Although an increasing proportion of resistant strains may be expected in years to come, the rather slow emergence of such strains is somewhat surprising when one considers results obtained in the study of *E. coli*. Smith and Crabb(6) noted very high proportions of tetracycline resistant *E. coli* in pigs fed the substances. Buttiaux *et al.*(2) noted that among cultures of *E. coli*, type 111:B_4, isolated from children admitted to one hospital the percentage of resistant strains rose from zero to 96.2 over a 2 year period. These findings suggest that *S. typhimurium* develops resistance to tetracyclines much more slowly than does *E. coli*.

Summary. Cultures of *Salmonella typhimurium* isolated from man and animals prior to 1948 and cultures of the same serotype isolated from man and fowls after Jan. 1956 were compared for resistance to several antibiotic substances. Significant results were obtained only with tetracyclines. Whereas none of the 200 older cultures examined was resistant to tetracyclines, 9% of cultures recently isolated from fowls and 5% of cultures recently isolated from man were resistant to the substances. Attention is called to the possible relationship of these findings to the inclusion of tetracyclines in poultry feeds.

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Effects of 5-Hydroxytryptamine on Some Aspects of Hemorrhagic State in Radiation-Induced Thrombocytopenia.* (23802)

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The presence of serotonin in platelets has been related to their vasoconstrictor activity (1). The connection of this activity with hemostasis was investigated in a number of clinical studies(2,3,4). Experiments under standardized conditions, however, were confined to intact animals(5,6,7). Since disturbances of hemostasis emphasize the effects on the hemostatic mechanism, the action of serotonin on the bleeding in thrombocytopenia was investigated.

The effects of serotonin on increased vascular fragility and traumatically-induced bleeding in irradiated, thrombocytopenic animals are reported.

Methods and materials. 5-hydroxytryptamine-creatinine sulfate (Antemovis†) was used throughout these studies and was administered intraperitoneally to mice and guinea pigs. A 250 kv source with 0.25 mm copper and a1 mm aluminum filter was used for whole body X-irradiation of mice and guinea pigs. A dose of 325 r was given to the guinea pigs representing an LD_{50-60}, while 750 r (LD_{90-100}) was given to adult white Swiss-Webster mice. Vascular fragility was determined on the skin of guinea pigs by the negative pressure method(8). The number of petechiae formed under a negative pressure of 120-150 mm applied for 30 seconds was recorded. The time of bleeding induced by tail snips (approximately 1 mm from the tip) in Swiss-Webster mice was determined. All experiments were carried out in the period of maximal thrombocytopenia (6th-7th day for the guinea pigs and 7th-8th day for the mice).

Results. A. *Vascular fragility in irradiated, thrombocytopenic guinea pigs.* Vascular resistance of guinea pigs rendered thrombocytopenic by X-irradiation, was increased by the use of relatively large amounts of serotonin (Table I). Intravenous administration of the doses indicated in Table I produced marked pulmonary distress and occasional, transient convulsions lasting 2-5 minutes. The intraperitoneal injection of high doses of serotonin led to milder pulmonary reactions. Vascular fragility was affected only by doses of 5-hydroxytryptamine which were high enough to also produce pulmonary reactions. The type of response produced with 0.5 mg serotonin given intravenously is illustrated in Fig. 1.

B. *Duration of traumatic bleeding in irradiated, thrombocytopenic mice.* In these experiments serotonin was administered intraperitoneally in order to avoid local, vasospastic effects on the tail veins of the animals. Convulsions or respiratory distress were not observed. High doses of serotonin reduced the duration of bleeding (Table II). In the lowest effective dose range (0.25 mg) there was considerable variation in response. Shortening of the bleeding time was also observed in a control group of normal mice (Table II).

Discussion. Previous studies(5,6,7) have shown that 5-hydroxytryptamine increases vascular resistance and shortens the bleeding time in normal animals. This is consistent with the effects observed in thrombocytopenic animals in the present studies. The effective doses, however, for both normal and thrombocytopenic animals were high. The rate of administration of serotonin was found to be an important factor. When an effective dose (1 mg) was divided and given in 3 separate injections at 10-minute intervals, vascular resistance in guinea pigs was only slightly increased. It has been suggested(2) that serotonin reaching the circulation from the venous side may be removed by the lungs. It is possible, therefore, that the rapid administration of a large amount of this agent raises the

* This investigation was supported in part by AEC, Nat. Cancer Inst., N.I.H., USPHS Grant, and Children's Cancer Research Fn.

† Vismara terapeutici, Casatenovo, Brianza (Como), Italy.

TABLE I. Effects of 5-hydroxytryptamine on Vascular Resistance to Negative Pressure in Irradiated, Thrombocytopenic* Guinea Pigs.

No. of animals	Material	Dose	Route of admin.	Avg No. of Petechiae: Prior to inj.	10′ after inj.	30′ after inj.	60′ after inj.
20	saline	.2 cc	Intrav.	23 (19-26)†	21 (17-23)	24 (20-27)	20 (18-23)
25	serotonin	1 mg	Intrav.	21 (18-23)	2 (0- 5)	8 (1-12)	17 (12-21)
20		.5 "	"	26 (22-30)	7 (0-12)	9 (3-16)	29 (26-35)
18		.1 "	"	19 (17-21)	18 (16-23)	20 (17-25)	22 (18-25)
24		1 "	Intraper.	25 (21-29)	4 (0- 9)	16 (8-26)	28 (24-31)
15		.5 "	"	34 (31-38)	16 (11-28)	23 (18-32)	31 (28-34)

* Platelet counts less than 20,000/mm³. † Range of individual determinations.

TABLE II. Effects of Serotonin on Large Vessel (Tail-Snip) Bleeding of Normal and Irradiated Mice.

No. of animals: Irradiated*	Normal	Agent	Dose	Route	Avg time of bleeding (min.): Irradiated animals	Normal animals
75	25	saline	.2 cc	Intraper.	37 (32-43)†	9 (7-13)
30	10	serotonin	1 mg	"	4 (3- 7)	3 (2- 4)
36	20		.5 "	"	7 (4-10)	5 (3- 9)
50	20		.25 "	"	22 (14-36)	8 (4-14)

* Platelet count less than 40,000/mm³. † Range of individual determinations.

blood level above the threshold for pulmonary removal, thus permitting the excess to enter the arterial circulation and to exercise a generalized effect on the vascular bed. It should be noted also that the effects observed in our studies were of short duration.

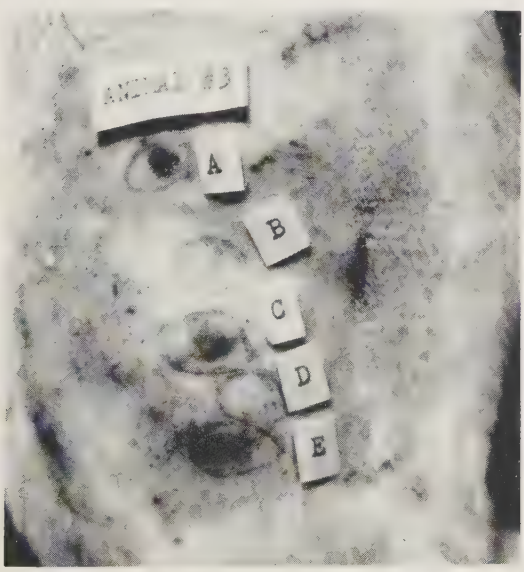

FIG. 1. Effect of intrav. serotonin admin. on vascular fragility of thrombocytopenic guinea pigs. A, C, E = Petechiae formed by negative pressure prior to inj. of serotonin. B, D = Tests 10′ following intrav. admin. of 0.5 mg serotonin.

Clinical investigations of serotonin have been confined to administration of relatively small doses(3,4). Clinical studies, which would be analogous to the animal studies reported here, have not been reported. Before effective doses and optimal modes of administration can be studied in man, the relation of hemostatic activity to toxic manifestations will have to be evaluated further. It should be noted, in this connection, that renal damage was not observed with the doses required for hemostatic effects in this study. The data presented here suggest that further clinical evaluation of serotonin as a hemostatic agent may be warranted, should the administration of higher doses prove feasible.

Summary. Synthetic serotonin (5-hydroxytryptamine-creatinine sulfate) increased vascular resistance and shortened the duration of traumatic bleeding in irradiated thrombocytopenic guinea pigs and mice, respectively. These effects were of short duration and were observed only when large doses of serotonin were given rapidly. Transient pulmonary distress was associated with the administration of effective doses to guinea pigs.

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Anaphylaxis in Guinea Pig: Improbability of Release of Serotonin in the Schultz-Dale Reaction.* (23803)

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Much knowledge concerning anaphylaxis has been gained from experimentation using the guinea pig, where it repeatedly has been shown that most symptoms of anaphylaxis are referable to release of histamine. The methods often employed antihistaminic drugs, but as pointed out by Dale(1), and Hawkins and Rosa(2), these drugs have inconsistently blocked the reaction; and their specificity is often suspect. Also, biological assay methods to measure histamine could conceivably be detecting other similarly reactive substances. These findings, together with failure to incriminate histamine in the rat and the persistence of the Schultz-Dale reaction in guinea pig tissues made refractory to histamine(3) cast doubt on the histamine theory of anaphylaxis. Other substances which might be involved in addition to histamine have been discussed by Dragstedt(4). Recent work on another species, the mouse, has demonstrated the improbability of involvement of histamine in the anaphylactic reaction; and the probability of the release of 5-hydroxytryptamine (serotonin). Evidence supporting the serotonin hypothesis includes prevention of the Schultz-Dale reaction in the presence of the highly specific serotonin antagonist, lysergic acid diethylamide (LSD),† and the demonstrably antiserotonic drug, reserpine(6). Further, Weiser(7) has shown almost 100% inhibition of generalized anaphylactic symptoms with reserpine; and Fox *et al.* with LSD(8). Conflicting reports have appeared recently regarding the role of serotonin in guinea pig anaphylaxis. Herxheimer(9) has shown that, although the guinea pig responds to an aerosol of serotonin in a manner similar to its response to histamine, and desensitization to an antigen is accompanied by a parallel tolerance to serotonin, LSD does not alter the anaphylactic reaction obtained after inhalation of antigen. During the course of the present work, however, Geiger *et al.*(10) reported that the Schultz-Dale reaction and the reaction to serotonin, but not the reaction to histamine, could be inhibited with the drugs yohimbine, bufotenine and gramine.

The purpose of this work was to determine whether or not the Schultz-Dale reaction in the sensitized guinea pig could be prevented with the serotonin antagonists LSD and brom lysergic acid diethylamide (BOL)(11). Following the publication of Geiger's work, an attempt was made to confirm his findings.

Procedure. Fully grown guinea pigs of both sexes from various commercial sources were used. They were injected intra-

* This investigation was supported by grant from Continuing Research Fund, University of Colorado Medical School.

† Munoz(5) has claimed that LSD does not specifically block serotonin, but this claim was not substantiated by any reference. We have been unable to uncover any evidence that LSD in the concentration used in these studies antagonizes the action of any other known physiological substance on smooth muscle.

abdominally with 1 ml of a 10% protein solution—either whole dried egg white‡ or bovine serum albumin§—approximately 3 weeks before being tested. At time of testing, animals were sacrificed by blow on head; the uterus or ileum was removed immediately, washed with Tyrode's solution, and placed in this solution at room temperature until used. Whole sections of ileum or uterus, 1-1.5 cm long, were cut and attached to an ink-writing kymograph lever in an aerated 50-ml water bath maintained at 37-38°C. After obtaining the pattern of normal contractions, the antigen—either crystalline ovalbumin‖ (EA) or bovine serum albumin (BSA)— was added 15 μg/ml of bath fluid. This amount of antigen had previously been shown to be optimal for provoking maximal contraction in a sensitized guinea pig tissue, and almost invariably rendered the tissue desensitized upon the second injection of antigen.

When it was definitely established that the tissue was sensitized, fresh strips were set up. Routinely, each strip was tested for its reactivity to serotonin¶ (0.1 μg/ml)**, and to histamine†† (0.1 μg/ml) prior to addition of antigen; a second addition of antigen was made to test for desensitization. In experiments testing drug inhibition of anaphylaxis, reactivity to serotonin and to histamine was determined; then the antagonistic drug was added for various periods of time. After addition of antigen to test for inhibition of anaphylaxis, a second addition of antigen was made to test for desensitization; lastly, reactions to standard amounts of serotonin and histamine were recorded. In any case where tissue failed to react after addition of an inhibitory drug, the potential reactivity of the tissue was tested by addition of 0.1 μg/ml of acetylcholine.

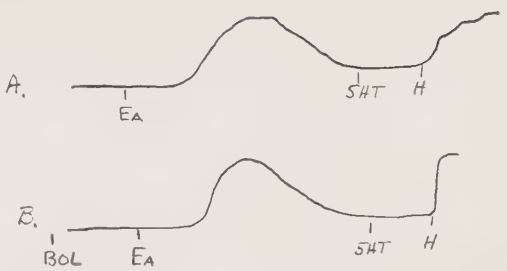

FIG. 1. Persistence of anaphylactic contraction of egg sensitive guinea pig ileum in the presence of BOL.* A—no drug present. B—BOL, 250 γ/l for 15 min.

* This tissue did not respond to serotonin.

Results. Experiments with LSD and BOL‡‡ included the use of 6 uteri from egg-sensitized female guinea pigs; the ilia from 6 egg-sensitized male guinea pigs; and the ileum from one male guinea pig sensitized with BSA. Following the routine as outlined previously, the reaction to antigen was tested in the presence of LSD in concentrations of 3 and 30 μg/L for 5 minutes; and in the presence of BOL in concentrations of 5, 50, 250 and 500 μg/L for 5 to 30 minutes. The LSD was allowed to remain in the bath during the addition of antigen; as recommended by Sollers *et al.*(11)§§, the BOL was washed out by replacing the fluid in the bath three times prior to the addition of antigen. All concentrations of BOL and LSD demonstrably inhibited serotonin. In no case was the reaction to antigen or to histamine modified. A typical response is represented in Fig. 1.

Experiments with yohimbine, bufotenine, and gramine.‖‖ To make these experiments more nearly comparable to Geiger's(12),

‡ Albumin, Egg Impalp. Powder, J. T. Baker Chem. Co.

§ Armour and Co. powder.

‖ Armour and Co.

¶ Serotonin creatinine sulfate, Nutritional Biochemicals Corp., Cleveland. Concentration calculated on weight as 5-hydroxytryptamine.

** Occasionally, a strip of tissue was found which did not respond to serotonin at this concentration, or at twice this concentration. These strips did, however, contract on the addition of antigen and of histamine.

†† Histamine phosphate, Parke Davis and Co.

‡‡ We are indebted to Dr. Harry Althouse, Sandoz Pharmaceuticals, San Francisco, for generous supplies of LSD, BOL, and bufotenine.

§§ These authors report that LSD does not antagonize serotonin in similar preparations in the guinea pig. For this reason, even though serotonin was demonstrably inhibited in these studies, more emphasis was placed on the work with BOL.

‖‖ We are indebted to Dr. W. B. Geiger for yohimbine (Amend Drug and Chemical Co. preparation); and gramine, from the Bios Labs.

the routine technic was modified by omitting magnesium from the Tyrode's solution; and the crystalline egg albumin was dialyzed against Tyrode's solution for 24 hours prior to use. Geiger allowed 15 minutes for contact of tissue with the inhibitory drug; both 15 and 30 minutes were used in this study. Variations from Geiger's technic included the use of histamine in a concentration of 0.1 μg/ml instead of 2 μg/ml; and antigen 15 μg/ml instead of 0.2 μg/ml. The small amount of histamine was used because it invariably provoked maximal contraction of the tissue; the larger amount of antigen was used because it was necessary to provoke maximal contraction of the tissue, and to lead to desensitization of the tissue.

Geiger found that the 3 drugs, in concentration of 20 μg/ml, prevented stimulation by antigen and by serotonin, but not by histamine. The 3 drugs, in concentrations of 20, 40 and sometimes 80 μg/ml were used in this study. Ilia from 3 animals sensitive to BSA and 2 sensitive to EA were tested in various concentrations of yohimbine; gramine was tested on ilia from two sensitized with BSA and two with EA; and bufotenine, on three animals sensitized to EA. Yohimbine in concentrations of 20, 40 and 80 μg/ml blocked serotonin contractions completely, with anaphylactic and histamine contractions being progressively diminished until made extinct at higher concentration of the drug. A typical response is presented in Fig. 2.

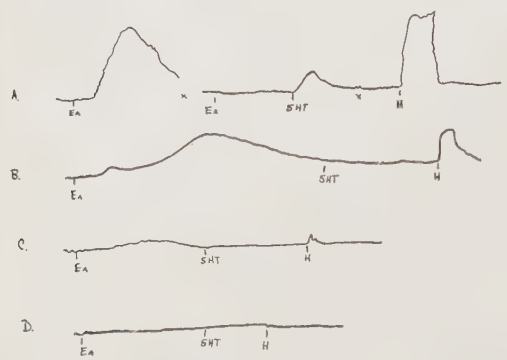

FIG. 2. Parallel effect of various conc. of yohimbine on the response of egg sensitive guinea pig ileum to antigen and to histamine.*

* A, no yohimbine; B, 20 γ/ml; C, 40 γ/ml; D, 80 γ/ml. Ea = 15 γ/ml dialyzed crystalline Ea. 5Ht = 0.1 γ/ml serotonin. H = 0.1 γ/ml histamine.

Bufotenine reacted similarly to yohimbine, inhibiting the serotonin contractions at 20 and 40 μg/ml, diminishing the histamine and anaphylactic contractions at 20 μg/ml, and abolishing them at 40 μg/ml.

Concentrations of gramine inhibiting the Schultz-Dale reaction inhibited both the serotonin and histamine reactions.

These reactions were consistently demonstrated in all tissues with all drugs tested.

Miscellaneous experiments. Additional experiments done to illustrate release of serotonin from sensitized guinea pig tissue were of 2 kinds: (1) These experiments employed the general technic of Campbell(13). With normal mouse uterus connected to the kymograph lever, an attempt was made to cause this highly serotonin-sensitive tissue to contract by adding to the bath: (a) perfusion fluid from EA-sensitized guinea pig lung perfused with antigen; or (b) whole sensitized guinea pig uterus, ileum or lung, plus antigen. Some of these experiments were conducted with Marsilid¶¶ 200 μg/ml in Tyrode's solution, to prevent the possible enzymatic destruction of serotonin(14). In no case was a contraction of the mouse uterus obtained. (2) Strips of sensitized guinea pig uterus were made insensitive to histamine by contact with high concentrations of this drug for long periods of time, then tested for reactivity to antigen. An attempt was made to block the persisting anaphylactic contraction with known inhibitory concentrations of BOL and LSD. In no case was the reaction even diminished.

Discussion. Using the Schultz-Dale technic as used previously to demonstrate the complete inhibition of *in vitro* anaphylaxis in the sensitized mouse uterus by the potent and highly specific serotonin antagonists, LSD and BOL, it was impossible to block the reaction in the sensitized guinea pig uterus or ileum. When the less specific serotonin antagonists yohimbine, gramine and bufotenine were used, and in much greater concentration, it was possible to block the reaction to serotonin, but the persisting reaction to antigen was entirely comparable to that of histamine. As the con-

¶¶ We wish to thank Dr. R. J. Floody, Hoffmann-LaRoche, Inc., Nutley, N. J., for the Marsilid (1-isonicotinyl-2-isopropylhydrazine) used.

centration of the inhibitory drugs was increased to the degree that complete inhibition of anaphylaxis occurred, there was complete inhibition of the reaction to histamine. These results are not in agreement with those of Geiger *et al.*(10), who demonstrated the blocking of the anaphylactic and serotonin contractions, but not the histamine contraction, with these drugs. They used much larger concentrations of histamine and less of antigen to provoke the reaction. It is possible that the histamine dosage used by Geiger was sufficient to overcome the blockade with the inhibitory substances; and that his antigen concentration might not have been sufficient to permit maximum reaction of the tissue.

No evidence for the release of serotonin was obtained by allowing various tissues from sensitized guinea pigs to be in contact with antigen in the presence of the highly serotonin-sensitive mouse uterus. Weissbach *et al.*(15) have reported that the serotonin content is low, and the monoamine oxidase activity high in the guinea pig lung. The low content might account for the failure to demonstrate serotonin release from the sensitized lung even in the presence of an amineoxidase inhibitor. The serotonin content of the small intestine, however, is greater(16), and had serotonin been released from the quantity of ileum used in these studies, it should have been sufficient to provoke a contraction of the mouse uterus, especially with the enzyme inhibitor present.

The persistence of the positive reaction to antigen in the guinea pig ileum or uterus poisoned to histamine is well known(3), and has been used as evidence against the histamine theory of anaphylaxis. Inasmuch as this reaction could not be modified with LSD or BOL, no argument can be made that this could be due to serotonin release. Thus the contention that the reaction is due to the release of intrinsic histamine which is not affected by poisoning with extrinsic histamine, cannot be challenged on these grounds.

The results obtained in this study tend to rule out the participation of serotonin in the anaphylactic contraction of smooth muscle in this species. The opposite situations prevailing in the mouse and in the guinea pig, as demonstrated by use of the Schultz-Dale technic, make even more pertinent the thoughtful admonition of Dragstedt(4): "Nor, . . . is there good reason why the . . . tissue metabolites associated with the phenomenon of anaphylaxis should have parallel degrees of importance in more than one animal species."

Summary. 1. The Schultz-Dale reaction in egg white or BSA sensitized guinea pig uterus or ileum persisted in the presence of serotonin antagonists LSD and BOL. 2. Anaphylactic contraction of these tissues could be partially or completely blocked with varying concentrations of yohimbine, gramine and bufotenine, but the reaction to histamine was affected similarly. 3. It was impossible to demonstrate the release of serotonin from sensitized guinea pig lung, uterus or ileum in the presence of highly serotonin sensitive mouse uterus. 4. Persisting anaphylactic contraction of histamine-poisoned guinea pig uterus was not diminished in the presence of BOL or LSD.

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Plasma 17-Hydroxycorticosteroid Concentrations in Fasted Dogs Following Oral Administration of Hydrocortisone Esters. (23804)

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Previous studies by Collins(1) and others (2,3,4) have indicated that (a) the rates of gastrointestinal absorption of hydrocortisone (F) and certain F analogues are not appreciably different and (b) the higher and more prolonged elevations of plasma 17-hydroxycorticosteroid (17-OHCS) concentrations after F analogues result from a decreased rate of metabolism.

In the rat, oral administration of certain F esters results in an enhanced liver glycogen deposition activity after 7 or 24 hours(5), suggesting that esterification of F delays intestinal absorption and/or degradation of the hormone. This possibility has been evaluated experimentally in the dog by an examination of plasma 17-OHCS concentrations as a function of time following oral administration of several F derivatives.

Methods. Laboratory-trained, male, mongrel dogs weighing 15-20 kg were used in these studies. The animals were fasted for 16-18 hours prior to steroid administration and food but not water was withheld during the blood sampling period. Each of the dogs was given various F esters at a dose equivalent to 20 or 40 mg of F. Following administration of the drug, blood samples were collected from the external jugular vein without anesthesia at hourly intervals for 5 hours. Plasma 17-OHCS concentrations were determined by a modification(1,6) of the Nelson-Samuels procedure(7,8). Each animal was tested at 10-day intervals. The following esters were studied: hydrocortisone cyclopentylpropionate (FCP), hydrocortisone acetate (FAc), hydrocortisone α-ethylbutyrate (FEB), and hydrocortisone α-ethylisovalerate (FIV).

Results. In the present studies only free 17-OHCS in the plasma were measured. Previous work has shown that neutral F esters of the type discussed are separated from free 17-OHCS during Florisil chromatography (6).

To test for the presence of intact ester in the plasma, chloroform extracts of 30-40 ml of dog plasma, collected 1 hour after a 60 mg dose, were examined by paper chromatography in the benzene-formamide system(9). No ester was detectable in the samples. Similar observations in the human have been reported (6). With the methods employed, intestinal hydrolysis of the esters prior to absorption cannot be differentiated from rapid hydrolysis by blood and liver esterases following the absorption of the intact esters.

Mean plasma 17-OHCS concentrations as a function of time following oral administration of F and the F esters studied are shown in Fig. 1. At a 20 mg dose, the esters produced maximum plasma 17-OHCS concentrations which were 45-73% of the value observed after F. In no case at this dose did the rate of removal of 17-OHCS from the plasma appear to be slower after ester than after free alcohol administration. At the 40 mg dose, the esters produced maximum plasma 17-OHCS concentrations which were 20-49% of that observed after 40 mg of F and were equal to (FCP) or less than the maximum elevation following 20 mg of F. However, at the higher dose, the disappearance of plasma 17-OHCS following ester administration was considerably slower (except for FAc) than after F. These results indicate a slower rate of absorption of steroid after administration of the F esters studied than after F itself.

Maximum plasma 17-OHCS concentrations as a function of the dose are shown in Fig. 2. Over the range studied a linear response was observed for F. However, doubling the dose gave only about a 50% increase in maximum plasma 17-OHCS with FCP and an even smaller increase with the other esters. It appears that maximum absorption rate is reached at a much lower dose of ester than of F.

Discussion. Plasma 17-OHCS curves such as those presented (Fig. 1) represent the resultant of factors tending to increase plasma

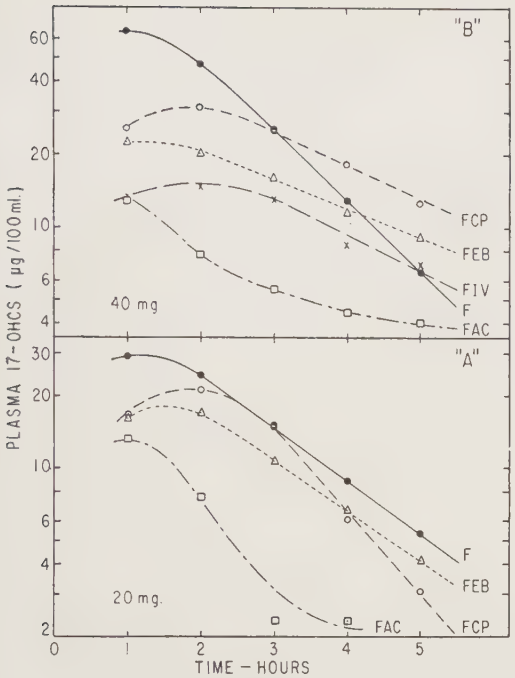

FIG. 1. Plasma 17-OHCS conc. *vs* time following oral administration of F and F esters.

levels, that is solution and absorption of the drug, and factors decreasing the levels, that is metabolism and excretion. Failure to detect intact esters in the plasma indicates that the free alcohol is the species metabolized. Therefore, any differences in the rate of disappearance of plasma 17-OHCS following administration of the F esters reflects a difference in absorption.

At the 20 mg dose, the lower maximum plasma 17-OHCS concentrations after F esters compared to F indicates a slower rate of absorption. At this dose of ester there is no alteration in the disappearance rate from the plasma from that observed with F (Fig. 1A). At the 40 mg dose, the slower rate of absorption is even more pronounced with the esters. In addition, the apparent disappearance rate of plasma 17-OHCS is reduced, compared to the rate after F, indicating continuing absorption from the gastrointestinal tract for a longer period of time (Fig. 1B). It appears that a maximum absorption rate is reached at a much lower dose with the esters than with F itself (Fig. 2). Increasing the dose does not greatly increase the maximum plasma 17-OHCS levels attainable but does maintain elevated 17-OHCS concentrations for a longer period. The rate of absorption of the F esters may be controlled by one or more of the following: (a) rate of solution of the esters, (b) solubility of the esters, (c) rate of enzymatic and/or non-enzymatic hydrolysis of the esters prior to absorption. The present experiments do not permit a choice among these possibilities. It may only be concluded that esterification modifies the intestinal absorption of orally administered steroid.

The efficient absorption of FCP is in agreement with the demonstrated clinical efficacy of this drug in the human(6). The poor absorption observed for FAc is as expected in view of the low order of activity of orally administered FAc in the dog(6).

Summary. Plasma 17-OHCS concentrations in the dog have been compared following oral administration of hydrocortisone, hydrocortisone cyclopentylpropionate, hydrocortisone acetate, hydrocortisone α-ethylbutyrate, and hydrocortisone α-ethylisovalerate. Prompt elevation of plasma 17-OHCS occurred within an hour after administration of any of these steroids. The magnitude of the response varied with the ester and dose but was always less than observed after an equivalent dose of hydrocortisone. At higher doses, the esters displayed a more prolonged elevation of plas-

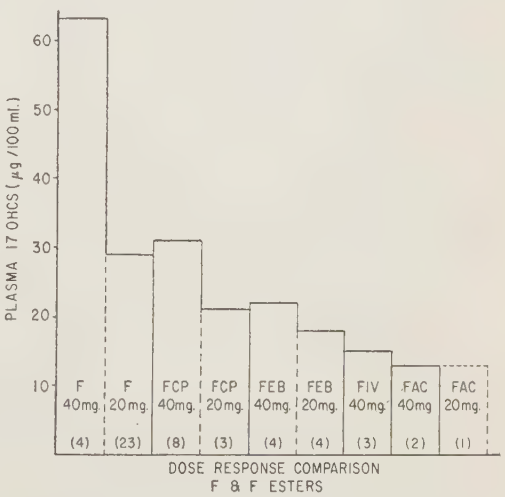

FIG. 2. Maximum plasma 17-OHCS conc. after oral administration of F and F esters. Results are avg of No. of animals indicated in parentheses.

ma 17-OHCS than found after hydrocortisone. In this respect, hydrocortisone cyclopentylpropionate was superior to other preparations studied. It is concluded that esterification alters the rate of steroid absorption.

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Teratogenic Changes in Early Chick Embryos Following Administration of Antitumor Agent. (Azaserine)* (23805)

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It has been previously reported that certain viruses, when inoculated in high concentration over the blastoderms of 48 hour chick embryos, produce specific teratogenic changes which are apparent on gross observation within 24 hours after inoculation(1-6). It has also been observed that antitumor agents often produce significant teratogenic changes in various embryonic organ systems. In recognition of the possible value of teratogenic studies in screening for antitumor agents, direct exposure of the frog embryo beginning at the 4-celled stage(7), and yolk sac inoculations of the chick embryo chiefly on fourth day(8), have been employed to determine teratogenic activity of various chemotherapeutic agents. The 2 methods, using different embryonic systems, however, have not always yielded comparable results for the same compound tested. It appeared that the 48-hour chick embryo, which consists of tissues in widely varying stages of proliferation with accompanying differentiation, might provide a sensitive testing system. If the changes should occur as rapidly as those following virus inoculations, this method would be particularly valuable in conserving time as well as laboratory space. Inoculations may be made directly over the cells of the developing blastoderm at this stage, thus avoiding possible interference by unknown variables such as absorption from the yolk sac, faulty access to susceptible organs via the circulatory system, or alteration of the compound *en route.* The substituted serine, O-diazoacetyl-L-serine (azaserine), having known antitumor activity (9), was selected for preliminary testing. This compound has previously been shown to produce developmental defects in the chick embryo following yolk sac inoculation at 4 days incubation(10), but produced less satisfactory results when inoculated into the yolk sac of unincubated eggs and eggs of 2 days incubation.

Materials and methods. The azaserine was obtained in pure form from National Institute of Health. As a control for specificity of action of the azaserine, experiments were also done using pure L-serine, obtained from Nutritional Biochemicals, Inc. For inoculations, the compounds were dissolved in saline buffered at pH 8.0. All materials used for inoculations were sterilized in routine manner except the compounds to be tested. The compounds were not sterilized because of the possibility of alteration by the process. Addition of antibiotics was omitted to avoid another variable. Broth cultures were made of all

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inocula, and occasionally of embryos dying after inoculation, but little difficulty was encountered from contamination. Fertile eggs, incubated in forced draft incubator at 99°F for 48 hours, were prepared by removing a square flap of shell from the side. The intact shell membrane was then flooded with saline. A slit was made down the center of the membrane to facilitate removal with forceps, care being taken not to injure the transparent vitelline membrane beneath. The blastoderm floats naturally at the top, and gentle rocking of the egg brings it into center of opening. The inoculum in .05 ml amounts of saline containing the various dosages was dropped onto the vitelline membrane directly over the embryo lying in center of blastoderm. The opening in the egg shell was then rimmed with a paraffin-vaseline mixture (1:3) and sealed with a flamed cover slip. After inoculation the eggs were incubated in a horizontal position with sealed area uppermost. Such embryos were in stages of development from 10-14 according to the criteria of Hamburger and Hamilton(11). Eggs receiving inoculations of saline only (pH 8.0) were included with each experimental group to determine whether other nonspecific teratogenic influences were present. Embryos were thereafter examined daily *in vivo* at 24 power magnification or harvested at specified intervals. Embryos harvested at 5 days incubation or less were fixed in Bouin's solution, the picric acid staining later removed in lithium-carbonate-alcohol. Embryos were stained overnight in very dilute aqueous solution of alum cochineal, cleared and examined while still floating in the clearing fluid. General growth inhibition was estimated by visual comparison with control embryos. General developmental retardation was determined by comparison of developmental stages with those of saline controls. Embryos were tabulated as showing specific organ defects if organs in question were retarded beyond that of the estimated developmental stage of embryo being examined, or if they were obviously small in relation to adjacent structures, or obviously different in structure from that of any normal stage. In searching for a dose of azaserine which might produce gross teratogenic changes within a short time without being lethal to embryos, a series of diminishing doses of azaserine was prepared in saline and inoculated into respective groups of eggs of 48 hours incubation. The embryos, examined *in vivo* 24 hours later, showed changes in the treated embryos, ranging from severe in group receiving the largest dose, to no visible changes in group receiving the smallest dose. The most obvious gross disturbance consisted of varying degrees of stagnation of blood cells in the smaller vessels of the circulatory system and varying degrees of hemorrhage. The heart continued to beat rhythmically, but circulation of blood appeared markedly impaired. In such embryos the extraembryonic blastoderms were small in comparison to controls. The embryos themselves appeared significantly different from controls. Exact changes were difficult to evaluate *in vivo*, so for fear that death might be imminent, all groups were harvested at 24 hours postinoculation for examination at low power magnification.

TABLE I. Effect of Various Doses of Azaserine on 12 Chick Embryos per Series, Inoculated at 48 Hour Incubation and Harvested 24 Hours Later.

		Teratogenic changes observed		
Dose (mg)	Surviving at 24 hr	General growth inhibition	Mean stage of development	Surviving embryos with specific defects
				%
.2	6	moderate	16.2	4 66
.1	7	"	15.3	7 100
.05	11	slight	15.4	9 82
.025	9	"	16.0	8 89
.013	11	none	16.4	7 64
.007	11	"	17.2	0 0
saline	11	"	17.1	0 0

Results. From the results shown in Table I, it is clear that azaserine in dosages of 0.012 mg or more produced general growth inhibition, general developmental retardation, as well as defective development in specific organs of the embryo (Fig. 1, 2).

It was found that as little as 0.06 mg of azaserine caused agglutination and hemolysis *in vitro* at 37.5°C when dissolved in 1 ml of a 0.5% suspension of washed erythrocytes from 17-day-old chick embryos. In view of possibility that agglutination and hemolysis *in vivo* were responsible for circulatory dis-

turbances, it appeared that the teratogenic changes observed might be secondary to the ischemic conditions produced. Circulation in the chick embryo is not established before stage 12. Therefore, studies were made to determine the effect of azaserine upon the embryo prior to this stage. Embryos of 24 hours incubation were in various stages of development, but none were advanced beyond stage 7. Exact staging of such young embryos was difficult *in vivo,* but presumably some were still in the streak stage. It is known that mechanical injury to vitelline membrane during this stage results in abnormal development of the embryo. This may account for the rather high incidence of abnormal embryos among controls. Controls inoculated with saline and harvested 24 hours later yielded 9 abnormal out of 22 embryos inoculated. However, among embryos inoculated with 0.025 mg azaserine and harvested 24 hours later, 25 out of 27 showed general retardation and abnormal development. More specific evidence that azaserine exerts a direct inhibitory effect was found, however, among embryos inoculated at 48 hours incubation, when an occasional embryo escaped gross circulatory disturbances but still suffered definite growth inhibition, developmental retardation and specific defects.

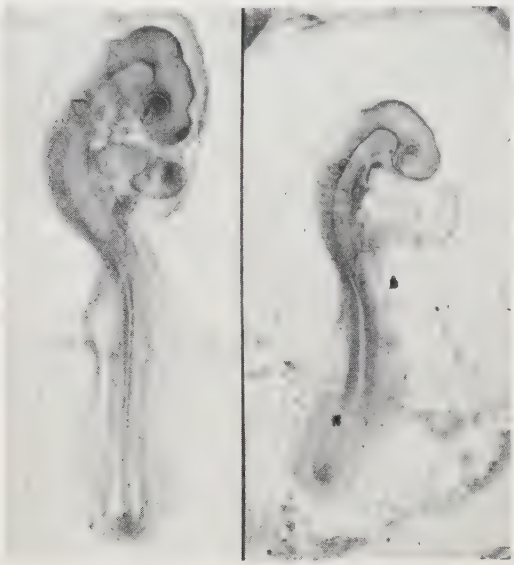

FIG. 1. Chick embryos of 72 hr incubation (×23). Left: Appearance of control embryo of avg size and development. Right: Appearance of embryo treated with azaserine 24 hr previously, showing characteristic growth inhibition and developmental retardation. Photographs by Visual Education Dept., Baylor Univ. College Medicine.

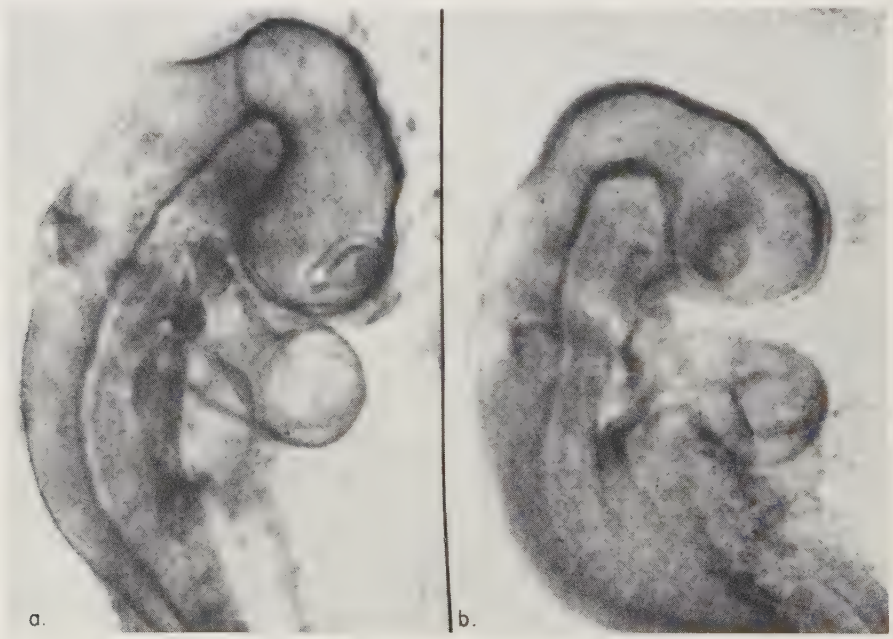

FIG. 2. Embryos of 72 hr incubation treated 24 hr previously with azaserine. a. Shows small lens. b. Shows retarded development of encephalon in region of forebrain and midbrain, including retarded development of optic cup and lens primordia.

TABLE II. Teratogenic Changes Observed in Embryos Harvested at Various Stages after Inoculation.

Inoculum	Harvest: Day after inoc.	Total eggs inoc.	Embryos surviving at harvest	No. surviving abnormal embryos		Observed effects in surviving embryos							
						Growth inhibition	Mean stage develop.	Retard. enceph.	Retard. optic cup	Small optic cup	Small lens	Small otocysts	Micromelia	Other
				%										
Aza.—.05-1.0 mg	1	87	49	35	73	Marked	14.5	12	30	0	12	1	*	
Control—saline	1	43	39	1	3	None	16.9	0	0	0	0	0	*	Flattened encephalon
Aza.—.05 mg	3	38	22	18	83	Marked	19.8	6	3	13	1	3	7	
Control—saline	3	10	10	0	0	None	25.5	0	0	0	0	0	0	
Aza.—.05 mg	5	37	16	11	70	Moderate	28.0	1	*	2	0	2	11	
Control—uninoc.	5	18	13	1	8	None	29.0	0	*	0	0	0	1	
Aza.—.05 mg	19	46	4	2	50	Marked		*	*	0	0	*	2	

* Stage at examination not conducive to gross observation of this defect.

To study survival of embryos as well as types of teratogenic changes found in embryos surviving various intervals after treatment with azaserine, groups of eggs were inoculated with 0.05-0.1 mg of the compound. Daily observation revealed that embryos with severe circulatory disturbances did not always die, but in many instances sustained re-establishment of circulation. Such embryos remained severely retarded and abnormal in development. From results shown in Table II, it is clear that teratogenic changes are seen in high percentage of surviving embryos up to time of hatching, although few reached this stage and none hatched. The encephalon, eye primordia and limb buds appeared more susceptible to inhibitory effects than did other tissues. The auditory vesicles were retarded rarely, but occasionally they showed unusually pointed appearance at site of attachment to body ectoderm. Exact staging was difficult in many embryos because the tissues tended to be blurred and took stain poorly. Some organs, particularly the auditory vesicles, were often seen to be developed beyond the stage assigned to the embryo through the staging criteria used. Since these organs were not developed beyond their counterparts in the control group, it was assumed they had escaped the inhibitory effect suffered by the rest of the embryo and represented more truly the stage the embryo should have attained. There was some shift in pattern of defects observed at different stages. However, certain defects are observed more readily at some stages than others. For instance, limb bud retardation can not be observed before stage 17, since limb buds are not sufficiently developed before this stage; and optic cups, after stage 19, have passed beyond the point where mild retardation of invagination can be observed grossly. Saline controls did not exhibit a significant number of abnormal embryos, and unsubstituted L-serine inoculated in doses of 0.2 and 0.05 mg failed to show any teratogenic changes when examined 1 day and 3 days after inoculation.

Discussion. Tissues during early embryonic life undergo proliferation at a greater rate than tissues of the fully developed individual, or even than tissues of the later em-

bryo. Furthermore, for each organ there are specific periods of increased proliferation at the time of beginning differentiation, presumably associated with altered metabolic activity. It is well known that tissues in such 'critical' states are more susceptible to the action of teratogenic agents than during less active periods. The chick embryo between stages 10 and 14 (approximately 48 hours incubation) is probably at the optimal stage for studies of teratogenic activity, using the technics described, since the inoculated material gains direct access to many organs in critical states of differentiation from the body ectoderm(2,3). Embryos younger than this do not show sufficient organ differentiation and do not survive the inoculation procedures as well. In embryos older than this the gross changes are not as striking, and the harvested embryos are more difficult to examine. It is interesting, therefore, that azaserine treatment of embryos at this critical period resulted in general retardation of growth and differentiation as well as specific retardation of organs which were in such critical stages of development, *i.e.* brain, optic primordia and limb buds. On the other hand, the auditory vesicles in similarly 'critical' stages were rarely affected. The reason for this is not known. However, organs undergoing differentiation doubtless differ significantly from each other, as well as from undifferentiating tissue.

Summary. The substituted serine O-diazoacetyl-L-serine (azaserine) was shown to produce teratogenic changes in the chick embryo when inoculated over the blastoderm at 48 hours of incubation. Such changes, observed on gross examination of harvested specimens within 24 hours after inoculation, consisted of general growth inhibition, general developmental retardation and specific retardation in the development of the encephalon, optic cup and lens vesicle. Examination of embryos allowed to continue incubation for longer periods of time revealed that teratogenic changes were present in a high percentage of the surviving embryos. Retardation of the encephalon was less apparent on gross examination of older embryos, but retardation of the limb buds was observed in high percentage of embryos examined from 3 days after inoculation up to the time of hatching. No gross changes were observed when control inoculations were made using saline or unsubstituted L-serine. The possible application of this technic in the study of antitumor agents was discussed.

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Effect of Ethylenediaminetetraacetic Acid on Radiostrontium Excretion in Man.* (23806)

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Certain metals which enter the animal or human body are rapidly deposited in the skeleton. This deposition can be counteracted by agents which form chelates with the metal and the metal chelate is then usually eliminated through the kidneys. The calcium salt of ethylenediaminetetraacetic acid (Ca-EDTA) is thus used as a detoxifying agent in poisoning with stable or radioactive metals, *e.g.*, lead, radioyttrium, and plutonium, since it has a higher affinity for these elements than for calcium(1-5). The sodium salt of ethylenediaminetetraacetic acid (Na-EDTA) may be even more effective for the removal of certain elements: it binds calcium and other metals in the extracellular fluid, causes demineralization and thereby inhibits the deposition of metals in bone and also promotes their removal from bone(6).

The rapid injection of Na-EDTA causes hypocalcemia and tetany(7). When this agent is infused slowly the calcium bound in plasma is replenished by calcium from bone to restore calcium homeostasis and the calcium chelate is excreted(6,8). Although EDTA binds calcium more strongly than strontium, it was of interest to study whether the demineralization caused by Na-EDTA interferes with the deposition of radiostrontium in bone, promotes the release of the already deposited isotope and thereby enhances the excretion of radiostrontium. The results of studies performed in three patients† with radioactive strontium (Sr^{85}) and Na-EDTA are reported in this communication.

Materials and methods. The investigations were divided into a control study which lasted 13, 13, and 18 days and, in an experimental study of 10, 10 and 5 days for the three patients respectively. A tracer of Sr^{85}, a γ-emitter (0.1-0.4 μc/kg) was injected intravenously on the first day of the control and experimental study in the fasting state.‡ Four grams of Na-EDTA were infused intravenously in 5% glucose in water over a 4-hour period on the first day of the experimental study and Sr^{85} was injected 30 minutes after the Na-EDTA infusion had been started. The Na-EDTA infusions were repeated on the two subsequent days in Patient 1 and on one day in Patients 2 and 3. Sr^{85} plasma levels, urinary and fecal excretions were determined during the entire study. Blood samples were obtained for Sr^{85} determinations at 1, 4, 8 and 24 hours following the injection of the tracer, daily for the next six days, and three times per week thereafter. The urine of the first day of each study was collected for radioassay in fractions at time intervals at which the blood samples were taken. Sr^{85} was then determined daily on 24-hour urine collections. The Sr^{85} content of each stool specimen was determined. The technic of the administration and of the measurement of Sr^{85} in blood and excreta has been described in previous publications(9,10). Metabolic balances of nitrogen, calcium and phosphorus were determined on six-day pools in the two phases of the study. The urinary calcium and phosphorus were determined in each urine fraction on the days of Sr^{85} administration and daily thereafter.

Results. Fig. 1 shows the results obtained in Patient 1. The rate of urinary radiostrontium excretion decreased considerably on the three days of Na-EDTA infusion and increased markedly thereafter so that the total excretion in 13 days was comparable in both studies, 39.7% versus 39.9%. The rate of urinary calcium excretion (lower half of Fig.

* This study was performed under Contract with U. S. Atomic Energy Com.

† Patient 1, 70 yr old male. Diagnosis: probable carcinoma of lung. Patient 2, 40 yr old female. Diagnosis: Hodgkins disease. Patient 3, 61 yr old male. Diagnosis: Carcinomatous polyp of colon.

‡ Produced by Nuclear and Engineering Corp., Pittsburgh, Pa.

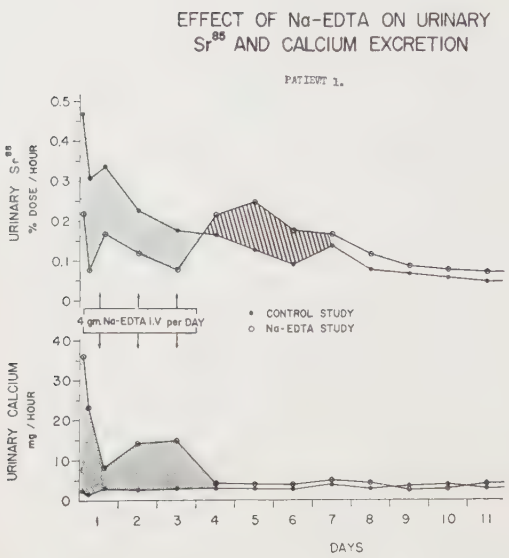

FIG. 1.

1) increased significantly on the three days of the Na-EDTA infusions, the excess calcium excretion being 70, 62 and 67% of the binding power of the chelating agent.

The effect of Na-EDTA on urinary calcium and Sr^{85} excretions of Patients 2 and 3 was similar to that of Patient 1: decrease of urinary radiostrontium and increase of urinary calcium excretion (Table I).

An attempt was made to estimate the ionized calcium excreted on the days of Na-EDTA administration by comparing the ratio of urinary Sr^{85}/Ca^{++} of the control to that of the experimental study:

$$Ca^{++}\ \text{exp.} = \frac{\%\ Sr^{85}\ \text{exp.}}{\%\ Sr^{85}\ \text{control}} \times Ca^{++}\ \text{control},$$

assuming that the Sr^{85}/Ca^{++} ratio remains unaltered when Na-EDTA is infused. These calculations reveal that most of the calcium excreted on the days of Na-EDTA administration was chelated and that the urinary excretion of ionized calcium of each patient was lower than in the control study (Table I).

The plasma curve of each of the three patients was only slightly higher in the experimental than in the control phase and did not reflect the major changes of urinary Sr^{85} excretion. The clearances of calcium and Sr^{85} were calculated. The data of Patient 1 are illustrated in Fig. 2. In the control study, the clearance of Sr^{85} was approximately five times higher than that of calcium. In the Na-EDTA study, the Sr^{85} clearance decreased by a factor of 2-2.5 while the clearance of total calcium (Ca^{++} + chelated calcium) increased considerably. Following the discontinuation of Na-EDTA, the Sr^{85} and calcium clearances returned rapidly toward control levels, the Sr^{85} clearance being slightly higher than in the control phase.

The fecal Sr^{85} excretions did not signifi-

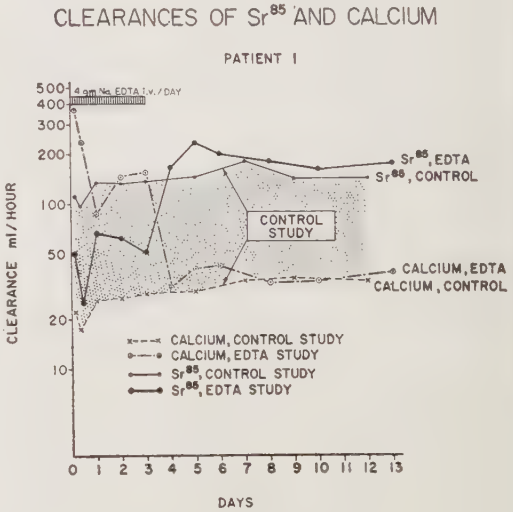

FIG. 2.

TABLE I. Effect of Na-EDTA upon Urinary Sr^{85} and Calcium Excretion.

Patient No.	Type of study	Urinary Sr^{85}, % dose Day 1	Urinary Sr^{85}, % dose Day 2	Urinary calcium, mg/day Day 1	Urinary calcium, mg/day Day 2
1	Control	8.5	5.6	58	65
	Na-EDTA	3.9	2.8	383 (27)*	346 (33)*
2	Control	21.4	10.4	202	172
	Na-EDTA	9.4	8.1	469 (89)*	463 (134)*
3	Control	7.8	3.6	71	45
	Na-EDTA	2.7	1.6	318 (25)*	341 (20)*

* mg Ca^{++} estimated by Sr^{85}/Ca ratios (see text).

cantly differ in the control and experimental phase.

Discussion. Since the binding capacity of EDTA is higher for calcium than for strontium (Log $K_{Ca} = 10.6$ and Log $K_{Sr} = 8.6$), strontium will not be appreciably chelated by Na-EDTA in the presence of the large excess of calcium in the serum. Thus, the serum ionic calcium is lowered by the infusion of Na-EDTA while the serum ionic strontium level is not decreased. Increased tubular reabsorption of ionic calcium may aid in restoring calcium homeostasis, and may be accompanied by a parallel increase in tubular reabsorption of ionic strontium. This explains the decrease of urinary radiostrontium excretion during the excretion of *chelated* calcium, in contrast with the enhancement of urinary radiostrontium excretion when an excess of *ionized* calcium is excreted(11).

The increased tubular reabsorption may be the cause of the slight rise in the plasma level of radiostrontium. It is possible, however, that another factor may also be contributory: demineralization induced by the chelating agent may retard the entry of radiostrontium into bone and may promote the removal of the already deposited isotope. Bauer *et al.* have shown that half of the dose of Sr^{90} present in the skeleton of rats four hours after intraperitoneal injection of the isotope is already fixed in the non-exchangeable fraction of the bone salt(12). Studies carried out with Sr^{85} in man have revealed that approximately one-third of the injected Sr^{85} is deposited in the skeleton three hours after the intravenous injection of the tracer. The infusion of Na-EDTA may, therefore, increase the strontium content of plasma and of "soft tissues" due to demineralization and increased tubular reabsorption of strontium. Strontium can then be released from these sites upon termination of the EDTA infusion. The rebound Sr^{85} excretion compensates for the inhibition in the Na-EDTA phase so that the cumulative radiostrontium excretion in the experimental study is comparable to, but not in excess of that of the control study.

Summary. The effect of sodium salt of ethylenediaminetetraacetic acid, Na-EDTA, upon radiostrontium (Sr^{85}) metabolism in man was investigated under controlled metabolic conditions. Urinary radiostrontium excretion was inhibited in each of 3 patients when Na-EDTA was infused. This depression was followed by excess excretion of the isotope after discontinuation of Na-EDTA. The mechanism of action responsible for this metabolic shift is discussed.

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Synthesis and Metabolism of Tritium-Labeled DL-Kynurenine.* (23807)

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Conflicting reports have appeared in the literature concerning the role of kynurenine in the conversion of the amino acid, tryptophan, to the vitamin, niacin. Kynurenine has been reported to stimulate excretion of N^1-methylnicotinamide in rats(1) and to possess growth-promoting activity when it replaces niacin in a diet(2,3). Conversely, it has also been reported that kynurenine does not support growth(4), or stimulate excretion of N^1-methylnicotinamide(5). *In vitro* work has shown that rat liver catalyzes the formation of the niacin precursor, quinolinic acid(6), when 3-hydroxy-kynurenine‡ was in the media, but not when kynurenine was present in the media(3). The failure of the liver to convert kynurenine to 3-hydroxy-kynurenine in these experiments was interpreted as a choice of the wrong tissue or lack of true conditions for the conversion. More recently, De Castro *et al.*(9,10) detected 3-hydroxy-kynurenine paper chromatographically and colorimetrically in the products of a liver mitochondria incubation in the presence of nicotinic acid, L-kynurenine and reduced diphosphopyridine nucleotide.

In the present work kynurenine labeled with tritium has been synthesized and administered to rats to obtain isotopically labeled products from the urine and more definitely establish the role of kynurenine in the tryptophan-niacin metabolic sequence. Labeled kynurenine was injected intraperitoneally into rats and labeled quinolinic acid and N^1-methylnicotinamide were isolated from the urine. The amount of unmetabolized kynurenine in the urine was also determined by carrier technic.

Methods. Preparation of the labeled DL-kynurenine. One gram of DL-kynurenine was exposed to 2.4 curies of tritium gas under 0.39 atmosphere pressure for several weeks. Prior to purification analysis of the DL-kynurenine showed it had an approximate specific activity of 290 mc/g or 60.32 mc/mM. This labeled DL-kynurenine was mixed with cold DL-kynurenine in a ratio of 1:283 and the mixture was recrystallized from 66% ethanol to constant specific activity. Chromatographic and radiographic analysis of this material showed only the D and L isomers of kynurenine. The recrystallized kynurenine was used for the animal experiments; it had an activity of 1.982 mc/g or 0.413 mc/mM, when analyzed by the gas counting method of Christman(11). *Location of tritium in the labeled kynurenine structure.* The benzene ring structure of the DL-kynurenine contained 69.6% of the tritium activity present in the molecule or 0.2875 mc/mM. This was determined by hydrolyzing the kynurenine to ortho-amino-acetophenone with alkali by the method of Spacek(12) and then preparing the ortho-acetamido-acetophenone derivative by the method of Leonard(13). In preparation of the ortho-acetamido-acetophenone derivative dilute acetic acid was the solvent present in the final step of the reaction. It is noteworthy that repeated suspension of the ortho-acetamido-acetophenone in dilute acetic acid (0.4 *N*) for periods of 18 hours showed no exchange of tritium out of the ring positions, such as has been observed on suspension in dilute mineral acids(14). *Animal experiments.* Male albino rats from Carworth Farms, weighing 220-237 g (raised on a stock diet), were transferred to 9% casein-sucrose diet previously described(15). After 5 or more weeks, the rats receiving this diet were housed in metabolism cages. Forty-eight-hour control urines were collected prior to the injection of labeled DL-kynurenine intraperitoneally. The kynurenine was dissolved in 2 *N* H_2SO_4 and the solution adjusted to approximately pH 6.0 with *N* sodium hydroxide. One

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‡ For the system of numbering the carbon atoms of tryptophan and its products, see(7,8).

rat received 1 mg, a second, 6.24 mg, and a third, 12.48 mg of tritium-labeled kynurenine. Urine samples were collected for two succeeding 12-hour intervals under toluene, and these samples were assayed for free nicotinic acid(16), quinolinic acid(17), and N^1-methylnicotinamide(18). Quinolinic acid was isolated from the urine of 2 rats by a carrier method previously described(19). N^1-methylnicotinamide was isolated as the picrate with carrier N^1-methylnicotinamide prepared in this laboratory(6). Unmetabolized kynurenine was isolated from the urine of one rat with the aid of carrier by the method of Kallio and Berg(1). All compounds isolated from the urines were purified and analyzed for tritium by the dry combustion and gas counting technic of Christman(11).

Results. Conversion of DL-kynurenine into nicotinic acid. Table I shows an increase in nicotinic acid excretion by all rats receiving the DL-kynurenine. The increases in nicotinic acid excretion over the control period for each rat were not proportional to the doses given, and they represent 0.76%, 0.96%, and 0.49%, respectively, of the 1, 6.24, and 12.48 mg injected doses. Compared with the results of Henderson, Koski, and D'Angeli(20) these values suggest a greater ability of this strain to convert DL-kynurenine to nicotinic acid or a more efficient utilization of DL-kynurenine, when it is injected at a low level.

Conversion of DL-kynurenine into quinolinic acid. On the basis of the excretion figures in Table I the rats receiving 1, 6.24, and 12.48 mg of DL-kynurenine excreted 2.5, 1.3 and 1.6%, respectively, of the injected compound as quinolinic acid. These quinolinic acid values also represent a greater percentage excretion of this metabolite than the results of Henderson *et al.*(20), but this difference may be a dosage effect. In Table II the activity due to tritium in the ring, *viz.* 287.5 μc/mM, is used for the calculations on rats 2 and 3, since the ring structure was the only part of the molecule appearing in the excreted quinolinic acid and N^1-methylnicotinamide. The data in Table II show that for the 6.24 mg and 12.48 mg rats the percentages of injected DL-kynurenine excreted as quinolinic acid were 1.31 and 0.95%, respectively. The fact that the specific activities of the isolated quinolinic acid samples (179 and 127 μc/mM) shown in Table II were less than the activity of the injected kynurenine (287 μc/mM) may have been the result either of an exchange of ring tritium for hydrogen during metabolic changes, or of dilution with inactive quinolinic acid formed from sources other than the labeled kynurenine. The results indicate, however, that the greater part of the excreted quinolinic acid was formed from kynurenine.

Conversion of DL-kynurenine into N^1-methylnicotinamide. Table I shows that rats receiving 1, 6.24 and 12.48 mg of labeled DL-kynurenine excreted amounts of N^1-methylnicotinamide equivalent per mole to 1.77, 0.77 and 0.43% of the administered kynurenine. These results agree with those of Kallio and Berg(1) in showing that administered kynurenine increases the excretion of N^1-methylnicotinamide. Isotope evidence that the excreted N^1-methylnicotinamide arises from kynurenine is provided in Table II. Here it is shown that rats receiving 8.625 μc and 17.25 μc of DL-kynurenine as tritium excreted 0.359 and 0.562%, respectively, of this tritium as N^1-methylnicotinamide.

TABLE I. Effect of DL-Kynurenine on Excretion of Nicotinic Acid, Quinolinic Acid, and N^1-Methylnicotinamide.

			Excretion per 24-hr period					
	DL-kynurenine inj.		Quinolinic acid, γ		Nicotinic acid, γ		N^1-methylnicotinamide, γ	
Rat #	mg	mM	Control	Post-inj.	Control	Post-inj.	Control	Post-inj.
1	1.00	.0048	44.61	64.46 (2.5)*	31.6	36.1 (.76)	22.4	34.7 (1.77)
2	6.24	.03	41.89	105.67 (1.3)	9.4	44.9 (.96)	5.8	37.2 (.77)
3	12.48	.06	54.4	213.38 (1.6)	24.4	60.6 (.49)	11.9	59 (.43)

* Figures in parentheses represent % of inj. kynurenine excreted as various metabolites, calculated from the difference between control and post-inj. excretions.

TABLE II. Twenty-Four Hour Excretion of Quinolinic Acid, N¹-methylnicotinamide and Kynurenine after Injection of Labeled DL-Kynurenine.

		Rat No. 1	Rat No. 2	Rat No. 3
Sp. activity of inj. kynurenine, μc/mMole	a	413.0 *	287.5 †	287.5 †
Kynurenine inj., mMole		.0048	.03	.06
Tritium inj., μc	b	1.982	8.625	17.25
Quinolinic acid				
Q-acid excreted, mMole	c		.00063	.00128
Carrier added, mg/mg urinary Q-acid			8492.0	6344.0
Sp. activity of excreted Q-acid, μc/mMole	d		179.1	127.6
Tritium excreted in Q-acid $\mu c = c \times d$	e		.1128	.1634
% of injected tritium $= \frac{100\ e}{b}$			1.308	.947
Ratio $= \frac{\text{Sp. activity of excreted Q-acid}}{\text{Sp. activity of inj. kynurenine}} = \frac{d}{a}$			.623	.444
N¹-methylnicotinamide				
N¹-amide excreted, mMole	f		.00022	.00034
Carrier added, mg/mg urinary N-amide			15763.0	5676.0
Sp. activity of excreted N-amide, μc/mMole	g		141.0	286.0
Tritium excreted in N-amide $\mu c = f \times g$	h		.031	.097
% of injected tritium $= \frac{100\ h}{b}$			.359	.562
Ratio $= \frac{\text{Sp. activity of excreted N-amide}}{\text{Sp. activity of inj. kynurenine}} = \frac{g}{a}$			.49	.995
Kynurenine				
Kynurenine excreted, mMole	i	.00033		
Carrier added, mg/mg urinary kynurenine		5376.0		
Sp. activity of isolated kynurenine, μc/mMole	j	412.0		
Tritium excreted in kynurenine $\mu c = i \times j$	k	.136		
% of injected tritium $= \frac{100\ k}{b}$		6.86		

* Total specific activity/mMole.
† Specific activity/mMole based on ring activity alone.

Almost all the activity in the kynurenine ring appears to be located in the 4, 5 and 6 positions, and practically none in the 3 position, as indicated by the following considerations. In tryptophan metabolism the 7 carbon of tryptophan, which becomes the 3 carbon of kynurenine, has been shown to be lost in the formation of N¹-methylnicotinamide (7) If a significant part of the tritium in the kynurenine used were bound to its 3 carbon, it would therefore presumably be lost in the formation of N¹-methylnicotinamide. However, the tritium activity of the N¹-methylnicotinamide (286.0) was the same as the ring activity (287.5) of the injected kynurenine, indicating no such loss. Wilzbach has shown that ring compounds are not uniformly labeled in all positions, when they are labeled by the gas exposure technic(21).

Kynurenine excretion. Rat No. 1 receiving one mg of DL-kynurenine excreted 6.86% of the tritium in unchanged kynurenine (Table II). If a similar percentage of the higher levels (6.24 mg and 12.48 mg) of kynurenine were excreted unchanged, less than 10% of the ring-located, injected tritium was excreted during 24 hours in kynurenine and its products, quinolinic acid and N¹-methylnicotinamide.

Summary. 1. DL-kynurenine was labeled with tritium by the Wilzbach procedure. 2. Intraperitoneal injection of tritium-labeled DL-kynurenine into rats was followed by the excretion of labeled quinolinic acid and N¹-

methylnicotinamide in the urine. The evidence is definite that kynurenine can serve as a precursor of quinolinic acid and N^1-methylnicotinamide. 3. During 24 hours after injection of 1 mg of labeled DL-kynurenine, 6.86% was excreted as unchanged labeled kynurenine.

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A Method for Production of Arrhythmias in the Isolated Rabbit Heart. (23808)

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Rosenblueth(1), Scherf(2), and Harris(3) have described methods for the experimental production of cardiac arrhythmias. These methods, performed on the intact dog *in situ,* are laborious and time consuming. The isolated heart offers a simple and inexpensive procedure for studying cardiac activity; therefore, a series of experiments were undertaken to determine whether it would be feasible to induce arrhythmias in the isolated rabbit's heart.

Materials and methods. Rabbit hearts were perfused by the Langendorff technic(4) with Locke-Ringer's solution prepared with double-distilled pyrogen-free water. The solution is oxygenated, maintained at 37.5° and perfused at 40 mm Hg. It has been found necessary to have both auricles distended by the perfusate, and this can be accomplished by tying off all vessels except the superior vena cava, which is partially ligated. Electrograms are recorded following an adjustment period of 15-20 minutes. Potential difference is measured by placing electrodes at various positions in the myocardium. In order to determine the type of arrhythmia produced, the number and position of electrodes are altered. When a constant pattern is obtained the

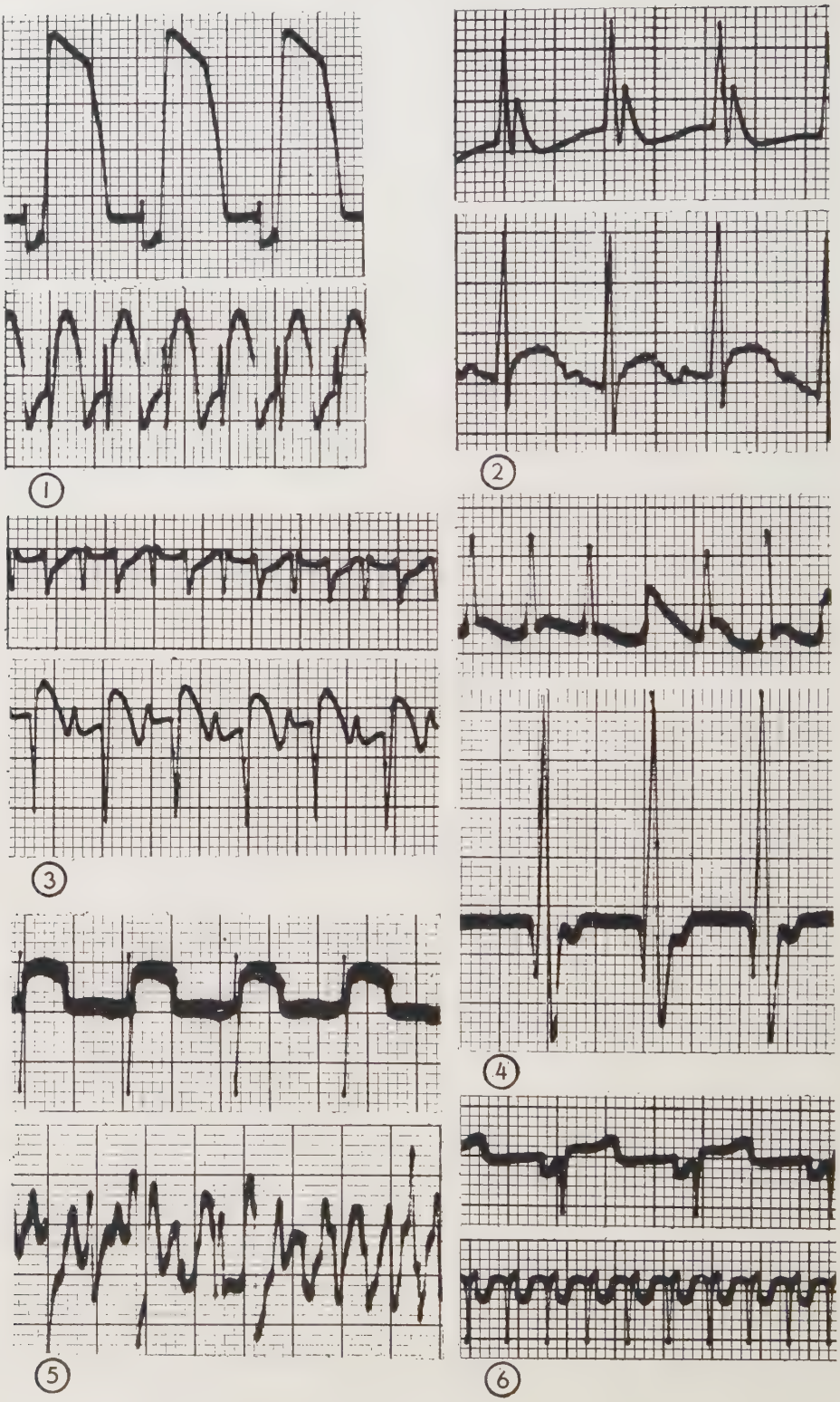
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FIG. 1. (Top) Normal electrogram (right auricle to left ventricle). Heart rate 116. (Bottom) Increased heart rate after electrical stimulation (tachycardia or flutter). Heart rate 236. Paper speed 25 mm/sec.

FIG. 2. (Top) Normal electrogram (right auricle to left ventricle). Heart rate 140. (Bottom) Right ventricle to left ventricle. Heart rate 140. Paper speed 25 mm/sec.

FIG. 3. (Top) Paroxysmal tachycardia instituted electrically. Right auricle to left auricle. Heart rate 216. (Bottom) Right auricle to left ventricle. Heart rate 216. Paper speed 25 mm /sec.

FIG. 4. (Top) A 2:1 auricular flutter instituted electrically. Right auricle to left auricle. Heart rate 498. (Bottom) Right ventricle to left ventricle. Heart rate 249. Paper speed 50 mm/sec.

FIG. 5. (Top) Normal electrogram (right auricle to left ventricle). Heart rate 138. (Bottom) Fibrillation pattern produced by electrical stimulation. Paper speed 25 mm/sec.

FIG. 6. (Top) Normal electrogram (right auricle to left ventricle). Heart rate 110. (Bottom) Increased heart rate after focal application of 0.05% aconitine nitrate. Heart rate 360.

cardiac rate is determined from the electrogram, and the heart is stimulated electrically. Electrical stimulation produces changes varying from paroxysmal tachycardia to atrial flutter and fibrillation. The same types of arrhythmia may also be produced by the topical application of 0.05% aconitine nitrate. An increased rate can be demonstrated by using two electrodes, embedding one in the right atrium and inserting the other in the tip of the left ventricle. The electrodes consist of two 12 mm lengths of enameled No. 30 copper wire which are soldered to Lead II electrodes of an electrocardiographic patient cable. After a normal electrogram is obtained, the right auricle is stimulated electrically for 5-10 seconds (5-10 volts, 50 pulses/second, pulse duration of 2-10 milliseconds, and pulse delay of 5 milliseconds). Fig. 1 shows a typical electrogram produced by this procedure. To differentiate between an atrial flutter and a paroxysmal tachycardia, 4 electrodes are placed in the myocardium. The 2 additional electrodes are soldered to an accessory shielded cable which is inserted into the input socket of the preamplifier on a Sanborn Twin-Viso Recorder. The potential differences are measured between the right and left auricles and the right and left ventricles. Fig. 2 shows a typical electrogram produced in this manner. Other leads may also be taken by varying the position of the electrodes. Fig. 3 and 4 respectively show the production of paroxysmal tachycardia and atrial flutter resulting from electrical stimulation. To demonstrate the induction of fibrillation, a normal electrogram is made by recording the potential difference between the right auricle and left ventricle. After the normal pattern is obtained the right and left auricles are rapidly stimulated alternately (140 volts, 50 pulses/second with a pulse duration of 2-10 milliseconds). Fig. 5 illustrates the fibrillation pattern produced in this manner. Focal application of 0.05% aconitine nitrate to the right auricle results in a tachycardia, atrial flutter, or fibrillation. Fig. 6 shows a tachycardia produced by this method.

Results. A total of 40 hearts was used to study the various arrhythmias discussed. Additional experiments were carried out to determine the duration of the induced arrhythmias. It was found that paroxysmal tachycardia and auricular flutter persisted for period varying 20 minutes to 6 hours, and at any time this could be reverted to a sinus rhythm by electrical stimulation. Further stimulation reestablished the arrhythmia. Once induced, a fibrillation would persist indefinitely.

Summary. A method has been presented for inducing experimental arrhythmias in the isolated rabbit heart. These vary from paroxysmal tachycardia to atrial flutter and fibrillation. Multiple leads from the myocardium are necessary in order to determine the type of arrhythmia produced. This method is inexpensive, rapid and the results are reproducible; therefore, we believe that this technic could be employed to advantage in screening eurhythmic agents.

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Esophagostomy in the Dog Allowing Natural Feeding. (23809)

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Esophagostomy and gastric fistula, introduced by Pavlov and Shumov-Simanovski(1) in 1890, has proved a useful preparation for the study of many aspects of gastric physiology, particularly since the introduction of a safe two-stage esophagostomy(2). However, the classical double-barrelled cervical esophagostomy makes the animal entirely dependent for survival on the care given it, and the difficulties encountered in maintaining the animal in a reasonably good general condition have proved a deterrent to the more widespread application of esophagostomy in experimental physiology. The present report proposes an esophagostomy designed to make the animal self-sufficient with regard to feeding and thus overcome the major difficulty of maintenance.

Technic. Esophagostomy is carried out in 2 stages. In the first stage the esophagus is exposed as described previously(2). The animal is anesthetized, and under strict asepsis a left antero-lateral cervical skin incision is made to extend longitudinally from level of cricoid cartilage to within 2 cm of suprasternal notch. The incision is deepened to pass between sternohyoid and sternothyroid muscles medially, and sternomastoid laterally. The deep cervical fascia is split, and the esophagus exposed after retracting the recurrent laryngeal nerve and sternohyoid and sternothyroid muscles medially, and the carotid sheath and overlying sternomastoid laterally. The rather well formed fascial layer investing the esophagus is opened along the length of skin incision. Localization and subsequent manipulation of esophagus is facilitated by intubation of esophagus with fairly rigid rubber or cardboard tube before surgery. The anterior and lateral aspects of esophagus are cleared by blunt dissection from its investing fascia; dorsal attachments, however, are left intact. Blood and nerve supply to exposed portion of esophagus are disturbed as little as possible. Two rows of interrupted stitches are inserted, a deep row to anchor the esophagus to the strap muscles and platysma, and a superficial one to exteriorize a 7-10 mm wide strip of anterior esophagus by suturing the superior, inferior and lateral aspects of esophageal muscularis to corium of the skin. It is essential to avoid penetration of the esophageal mucosa with these sutures. The esophageal tube is removed and the wound sealed with collodion. Penicillin and streptomycin may be administered for 2 days postoperatively. The second stage may be performed with safety any time after 4 days, but the most satisfactory period is probably between tenth and fourteenth day. The animal is anesthetized and the esophagus again intubated. Surgical asepsis is advisable but not essential during this stage. The skin incision is freshened and a 3 cm longitudinal incision is made through all layers of the exteriorized anterior esophageal wall. Esophagostomy is rendered permanently patent by suturing the muscularis and mucosa of esophagus to the skin. The esophageal tube is removed. Antibiotics may again be given for 2 days. Esophagostomy may shrink with time, but should this occur, it can be extended to the required length by incising upper and/or lower ends and suturing the incised esophageal and skin margins. This procedure can be carried out without anesthesia or aseptic precautions if the incision is limited to the scar in the skin and esophagus.

Effective separation of the lower from the upper portions of the esophagus for experimental purposes is achieved by forming a temporary flap of esophageal mucosa and bringing it out through the esophagostomy. This is done simply by attaching 2 or 3 small hemostats to posterior and lateral aspects of the esophageal mucosa at level of lower end of the esophagostomy. The weight of the hemostats is sufficient to evert the esophageal mucosa, and the preparation now assumes the advantages of the standard double-barrelled type of experimental esophagostomy. The application of hemostats for several hours twice weekly has not caused lasting compression in-

jury of the esophageal mucosa.

Discussion. This two-stage esophagostomy is a safe operative procedure, and the animals have been found to satisfy their nutritional requirements by the natural processes of eating and drinking. 70-90% of food swallowed passes directly into the stomach, and the portion which escapes through the esophagostomy, if accessible to the animal, may be eventually consumed.

Four dogs equipped with this type of esophagostomy have been maintained in good general condition for more than a year, and the time spent on their care was hardly greater than that required for dogs without esophagostomy. The 3 cm esophagostomy was small enough to make the animal self-sufficient with regard to feeding, and large enough to permit satisfactory eversion of a flap of esophageal mucosa in experiments requiring the exclusion of saliva from gastric secretions. It is of interest that duodenal reflux, as evidenced by the appearance of bile in the gastric collection, was very infrequent in all our preparations with a gastric fistula placed on the most dependent part of the anterior surface of the body.

In our experience, gastric fistula supplemented with the described esophagostomy provides the simplest and most satisfactory preparation for the collection of gastric secretion free of salivary contamination, and is eminently suitable for the study of secretagogue or inhibitory activities of various substances administered parenterally. This type of esophagostomy may also be utilized for mild or moderate stimulation of the cephalic phase of gastric secretion by "limited" sham feeding, a procedure whereby the animal is fed for a few minutes with small single strips of meat. The preparation, however, is less suitable for maximal cephalic phase responses by prolonged unrestricted sham feeding. The latter may be associated with esophageal movements sufficiently violent to retract the everted mucosal flap through the esophagostomy, and thus allow the passage of small amounts of food into the lower esophagus and stomach.

Summary. A safe two-stage operative technic is described for establishing an esophagostomy in the dog which permits natural feeding. When required for experimental purposes, a simple procedure allows this esophagostomy to assume, temporarily, the advantages of the standard double-barrelled esophagostomy.

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Production of Antibodies by Adult Hen Spleen Cells Transferred to Chick Embryos.* (23810)

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It is generally accepted that the chick embryo is incapable of producing antibodies(1). A wide variety of cells and tissues have been successfully grafted onto the chorioallantoic membrane (CAM) of the developing chick embryo. The purpose of the work reported here was to determine whether, and under what conditions, adult hen spleen cells, transferred to CAM, would produce antibodies.

Materials and methods. In all experiments Wynhamp or New Hampshire Red hens (ca 2 kg) were used as donors. Bovine serum albumin (BSA), Armour's Fraction V, in Hanks or in phosphate buffered (pH 6.4) saline solution (PBS), was the antigen used. The donor hens were killed by decapitation and the

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spleens removed aseptically. The spleen capsules were removed and the pulps pressed through surgical gauze (28x24 threads per inch). The pulp was placed in 3 ml of Hanks solution in graduated centrifuge tubes. The volumes of pulp obtained, as indicated by the final volume of fluid, ranged from 1.1 to 3.5 ml. These suspensions were used as such, or in some experiments the buffy coats were separated by centrifugation and the white cells resuspended to the original final volumes in Hanks solution. Portions of the suspensions were preserved for antibody determinations and 0.1-ml amounts were inoculated by syringe and needle directly onto the CAM's of varying numbers of 9- to 10-day-old embryonated Hyline (934A) eggs. The eggs were reincubated for various periods and the CAM's in the region containing the cells (ca 20 mm diameter) were removed. The fluids were expressed from the membranes and transplanted cells by tissue press(2) and collected in graduated capillary pipettes. The original spleen or white cell suspensions were ground in mortars with PBS. Antibodies in both types of fluids were determined by the hemagglutination technic of Boyden(3). Erythrocytes were sensitized by solutions of BSA containing 0.25 mg/ml. Specificity of the reactions was controlled by inhibition by specific antigen(4), using 0.2 mg BSA per ml of test fluid.

Results. Preliminary experiments were done to determine the possible distribution of antibody which might be formed on the CAM. High-titered anti-BSA rabbit serums were dropped onto the CAM's of 10-day-old embryos and the relative proportions of antibody recovered from the various tissues and fluids measured after incubation for periods of 1 to 6 days. The major portion of the antibody was, in almost all cases, found in the CAM's in the area of injection. The remainder of the CAM's contained about one-fourth as much as the local area, and the embryos, allantoic fluids, and amniotic fluids little or none. Similar results were obtained using an anti-BSA hen serum, but the recovery efficiency was not as great because the serum used was of low titer. Because of these findings, it was decided to limit the titration of antibodies to the fluids obtained from the inoculated cells and the CAM area involved.

While the literature given above is quite conclusive that embryonic tissues alone cannot produce antibodies, we took the precaution of determining, with the antigens and methods to be used, that no antibody would be produced by the embryonic tissues in the absence of transplanted cells. A total of 27 10-day-old embryonated eggs was inoculated on the CAM with 0.01 to 1 mg of BSA and incubated for 6 to 8 days. The CAM fluids were titrated but no trace of antibody was found in any.

Transfer of normal adult spleen cells to CAM's. Cells incubated with antigen before inoculation. Spleen suspensions from 4 normal donor hens were incubated *in vitro* with BSA (40 μg/ml of packed cells) for 30 minutes at 37°C. The cells were washed once with Hanks solution, and 0.1-ml amounts were inoculated onto the egg membranes. None of the transplants to the 31 surviving embryos showed any antibody after 4 to 7 days incubation.

Simultaneous inoculation of cells and antigen onto CAM's. 0.1-ml amounts of spleen suspensions from 6 normal hens were inoculated onto the membranes of embryos along with various amounts of antigen (4 μg-200 μg). Transplants on the 30 surviving embryos showed no antibody after 6 to 8 days incubation.

Transfer of spleen cells 1 to 2 days after one injection of antigen. No other antigenic stimulation. Thirteen donor hens were given 32 mg/kg of BSA intravenously, and the spleens removed after 2 days. Spleen suspensions were inoculated onto the CAM's of embryonated eggs, and the antibody titers of the fluids from the transplants and adjacent CAM's measured after varying periods of incubation. The results are summarized in Table I. Approximately one-third of the transplants showed definite antibody after 4 to 7 days incubation. Although the titers were variable, many of them were high enough to leave no doubt of their significance. No antibodies were detected after 2 to 3 days incubation. The original cell suspensions inoculated in no case showed any detectable

TABLE I. Antibody Production by Whole Spleen Transplants from Hens Given Intravenous BSA 48 Hours Previously.

No. of donor hens	Incubation time of eggs after cell transfer (days)	No. of transplants producing antibodies	Total No. of embryos surviving	Mean log titers of positives	Range of positive log titers
2	2	0	13		
1	3	0	5		
9	4	20	48	1.57	.70–2.95
9	5	14	38	1.75	1.08–2.76
5	6	6	20	2.17	1.26–2.89
3	7	4	12	1.81	1.20–2.48

Donor hens were given 32 mg BSA/kg body wt.
Transplants from individual donors were incubated for several different time periods. All spleen suspensions were devoid of detectable antibody at time of transfer.

antibody. Not included in the table are the results from experiments using 2 donor hens whose spleens were removed 24 hours after intravenous antigen injection and the recipient embryos incubated for 5 to 7 days. None of the transplants of the 17 surviving embryos showed any antibody in contrast to the one-third positives in the 5- to 7-day transplants from donors injected 48 hours previously, shown in the table.

In a second group of experiments using white cell suspensions from spleens removed 36 and 48 hours after intravenous antigen injection, definite evidence of antibody production was also found, as shown in Table II.

Transfer of spleen cells 2 days after reinjection of antigen. No other antigenic stimulation. One hen was given a total of 185 mg of BSA intravenously over a period of 33 days. Two days after the final injection of 20 mg, the spleen cells were transferred to eggs which were incubated for 2 to 6 days. Extracts of the spleen suspensions contained antibody (log titer 1.45). Transplants and adjacent membranes from 5 of the 10 surviving embryos showed antibody (log titer 0.78-1.75). In 2 cases (2 and 6 days incubation) the antibody recovered was significantly greater than the amount inoculated. One hen, given 20 mg BSA intravenously 12 days after primary intramuscular inoculation of 0.5 mg of alum-precipitated BSA, was killed 2 days later. The spleen suspensions contained no antibody. Four of the transplants on the 7 surviving embryos (2 to 6 days incubation) contained measurable antibody (range of log titer 0.60-1.90).

Transfer of spleen cells more than 7 days after one or more antigen injections. Cells incubated with antigen before inoculation. Spleen suspensions from 6 donor hens which had received one intravenous injection of 32

TABLE II. Antibody Production by Spleen White Cell Transplants from Hens Given Intravenous BSA 36 or 48 Hours Previously.

Time spleen removed after antigen inj. (hr)	No. of donor hens	Incubation time of eggs after cell transfer (days)	No. of transplants producing antibodies	Total No. of embryos surviving	Mean log titers of positives	Range of positive log titers
36	2	4	0	8		
	6	5	1	22	1.60	
	4	6	1	15	3.34	
	2	7	0	9		
48	6	4	8	23	1.94	1.26–2.76
	5	5	6	18	2.30	1.57–2.76
	4	6	3	13	2.39	1.90–2.81
	1	7	1	2	2.78	

Donor hens were given 32 mg BSA/kg body wt.
Transplants from individual donors were incubated for several different time periods. All white cell suspensions were devoid of detectable antibody at time of transfer.

mg/kg of BSA, 7 to 28 days previously, were incubated *in vitro* with BSA (40 μg/ml of packed cells) for 30 minutes at 37°C. The cells were then washed with Hanks solution, and 0.1-ml amounts inoculated onto the CAM's, and incubated for 2 to 5 days. Of 62 surviving embryos, 5 showed low antibody titers which undoubtedly represented only transferred antibody, since the spleen suspensions inoculated contained antibody. Calculation of antibody units inoculated and recovered indicated that there was no increase of antibody in the eggs. Similar experiments were done with 2 donor hens which had received two intravenous injections 30 and 15 days previously. The spleen suspensions were incubated as above with BSA, inoculated, and the eggs incubated for 1 to 3 days. Seven embryos survived, none of which showed any trace of antibody.

Simultaneous inoculation of cells and antigen onto CAM's. Six hens were given one intravenous injection of BSA, 32 mg/kg, and killed 27 to 40 days later. 0.1-ml amounts of spleen suspensions were inoculated onto the CAM's, and then varying amounts of BSA (4 μg-200 μg) were dropped onto the cells. The eggs were incubated for 5 to 7 days. None of the transplants on the 45 surviving embryos showed any trace of antibody. Two hens were given several intravenous injections of BSA (totalling 152 and 146 mg) and killed 68 and 265 days after the last injection. Eggs inoculated with the spleen suspensions and antigen (4 μg to 200 μg) were incubated for 5 days. None of the transplants on the 25 surviving embryos produced any detectable antibody.

Discussion. It was clearly demonstrated that antibodies were produced by the whole spleen or spleen white cell suspensions transferred to CAM's, without further contact with antigen, from adult donor hens 36 to 48 hours after a single intravenous injection of BSA. Extracts of the suspensions never contained demonstrable antibodies at the time of transfer, while in many instances the CAM-transplant fluids contained significant concentrations of antibodies. The possibility that the embryo tissues were enabled to produce antibodies by factors supplied by the adult cells, is made unlikely by the fact that no antibodies were demonstrated in the membranes onto which normal adult cells and antigen had been inoculated. Cells transferred 48 hours after *reinjection* of the adult donor hens also produced antibodies. While antibody was usually present in the suspensions transferred, the amounts of antibody recovered from the CAM-transplants were, in a number of instances, significantly greater than the amounts transferred. These results are in agreement with the numerous reports that cells, taken from adult animals at varying periods from 1 to 10 days after a single or final stimulation by various bacterial and protein antigens, have consistently produced antibodies, when transferred without further antigenic stimulation to homologous, normal or irradiated adults, to newborn animals, and to tissue cultures(5).

No evidence of antibody production was obtained in the experiments in which cells were taken from adult hens more than 7 days after one or more antigen injections and transferred to CAM's simultaneously with antigen or after *in vitro* incubation with antigen. These results are in agreement with those of Dixon and Weigle(6), who found that cells, removed from adult rabbits 17 to 21 days after a final injection of protein antigen, did not produce antibodies when transferred to newborn animals simultaneously injected with antigen. Such cells, transferred to x-irradiated adult animals, produced antibodies if the recipients were given antigen at the same time, but not if the cells were incubated with the antigen *in vitro* before inoculation(7).

Our failure to demonstrate antibody production by spleen suspensions from uninjected adult hens, when transferred to CAM's simultaneously with antigen, or after *in vitro* contact with antigen, is comparable to the previously reported results with normal cells(6). Cells from uninjected rabbits, transferred to newborn animals simultaneously with antigen, or after contact with antigen *in vitro*, failed to produce antibodies against protein antigens. Cells from donors injected with antigen 2 hours prior to removal, essentially normal cells in contact with antigen (?), failed to produce antibodies against Shigella antigen when

transferred to newborn animals. Cells from uninjected rabbits, transferred to x-irradiated adults simultaneously with antigen, or after contact with antigen *in vitro,* produced antibodies against Shigella antigens(5), but not against protein antigens(7). Cells from uninjected animals in tissue culture are generally recognized not to produce antibodies(5), but one report(8) has indicated that cells from animals non-specifically stimulated by endotoxin produced antibodies following an *in vitro* contact with protein antigen.

Summary. Spleen cell suspensions from normal hens inoculated onto CAM's with BSA, or after *in vitro* contact with BSA, failed to produce demonstrable antibodies. Whole spleen cell and spleen white cell suspensions, taken from previously normal hens 36 to 48 hours after intravenous injection of BSA and inoculated onto CAM's, produced significant amounts of antibody without further contact with antigen 4 to 7 days after transfer. The white cell suspensions produced somewhat more antibody than did the whole spleen suspensions. Whole spleen cell suspensions, taken from hens 48 hours after intravenous reinjection of BSA, produced significant amounts of antibody, without further contact with antigen, 2 to 6 days after transfer to CAM's. Whole spleen cell suspensions, from hens stimulated by BSA injection more than 7 days previously, inoculated onto CAM's with BSA, or after *in vitro* contact with BSA, failed to produce demonstrable antibodies.

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Cholesterol Esterases. VII. Hydrolysis of Branched Chain Esters by Pancreatic Cholesterol Esterase.* (23811)

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In a study(1) on the relative specificity and activity of hydrolytic and esterifying cholesterol esterase in pancreatic extracts in a series of 13 fatty acids, it was observed that the enzyme was most active in hydrolysis with short chain fatty acid esters, and most active in esterification with long chain acids. An interesting exception to the high hydrolytic activity with short chain fatty acid esters was found with cholesterol isovalerate. This 5-carbon branched chain ester was hydrolyzed at 9% of the rate observed with the n-valeric acid ester. This markedly lower activity with a branched chain acid suggested the possibility of inhibition of the enzyme owing to steric hindrance effects. It was also shown that in a mixture of 2 esters the more slowly hydrolyzed ester competes with the more rapidly hydrolyzed one for the enzyme. In this paper we present data on the hydrolysis of a series of cholesterol esters, of fatty acids of varying chain length with differing degree, location, and size of branching by pancreatic cholesterol esterase.

Preparation and characteristics of branched chain esters. The esters were synthesized as described by Swell and Treadwell(1) and were recrystallized until melting points were

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TABLE I. Physical Constants of Cholesterol Esters.

Ester	Molecular wt: Theoretical	Molecular wt: Found	Melting point, °C*	$[\alpha]_D^{20}$†
n-Butyrate	456.7	458.8	111.0	−35.4
Isobutyrate	"	467.3	124.5	−31.8
n-Valerate	470.9	471.7	90.0	−34.3
Isovalerate	"	463.0	111.0	−32.2
DL-Methylethyl-acetate	"	484.1	107.5	−33.8
Trimethylacetate	"	469.3	161.0	−34.6
n-Caproate	484.9	479.0	94.5	−32.6
Isocaproate	"	476.9	99.5	−34.4
2-Methylvalerate	"	492.3	103.0	−29.0
Diethylacetate	"	470.2	93.0	−32.8
2-Ethylcaproate			53.0	

* Clear melting point(1).
† Determined on 5% solutions in chloroform.

constant and they gave no precipitate with digitonin. For determination of molecular weights of these branched chain esters, it was necessary to modify the saponification method described by Jamieson(2). In preliminary determinations it was found that less than 50% of some esters was saponified in the 2-hour period specified by Jamieson. A time study of saponification of cholesterol diethylacetate, the most resistant ester in the series, showed that refluxing for 8 hours gave complete saponification. The modified method included use of 200 mg quantities of the esters, a comparable decrease in volume of the alcoholic potassium hydroxide solution, and an 8 hour period of saponification. The molecular weight, melting point, and specific rotation of each of the esters used are shown in Table I. *Determination of hydrolytic activity of cholesterol esterase.* Composition and preparation of substrate mixtures, preparation of the pancreatic extract, and conditions for assay of the hydrolytic activity were the same as described previously(1). Samples were removed for analysis at 0, 2, 4, 6, 12 and 24 hours of incubation. The 0 and 24 hour samples were analyzed for total cholesterol to insure uniformity of the digests during incubation, and all samples were analyzed for free cholesterol. The free cholesterol content was used for calculating the degree of hydrolysis and determining the linear portion of the time curve. The activity (mg of cholesterol liberated/g of tissue/hour) was calculated from a point, usually 2 hours, on the linear portion of the time curve. The Schoenheimer and Sperry method(3) was used for determination of free cholesterol in the digest, but for total cholesterol it was necessary to modify the saponification step in the method. An aliquot of the (1-1) acetone-alcohol extract of the enzyme digest in a 15 ml centrifuge tube was evaporated to dryness, and 2 ml of 6% potassium hydroxide in aldehyde-free absolute alcohol added. After thorough mixing, the tube was placed in an oven at 67°C for 8 hours, neutralized with 10% hydrochloric acid using one drop of phenolphthalein as indi-

TABLE II. Hydrolysis of Cholesterol Esters by Pancreatic Cholesterol Esterase.

Ester	Structure of acid	Hydrolytic activity*	Hydrolysis ratio†
n-Butyrate	C–C–C–COOH	335	100
Isobutyrate	C–C(C)–COOH	17	5
n-Valerate	C–C–C–C–COOH	353	100
Isovalerate	C–C(C)–C–COOH	29	9
DL-Methylethyl-acetate	C–C(C)–C–COOH	31	9
Trimethylacetate	C–C(C)(C)–COOH	1	0
n-Caproate	C–C–C–C–C–COOH	433	100
Isocaproate	C–C–C(C)–C–COOH	384	89
2-Methylvalerate	C–C–C(C)–C–COOH	14	3
Diethylacetate	C–C(C)(C)–C–COOH	7	2
2-Ethylcaproate	C–C–C(C)–C–C–COOH	1	0

* mg of cholesterol liberated per g pancreatic tissue per hr.
† Hydrolytic activity for ester/hydrolytic activity for ester of n-acid of same No. of carbons, × 100.

cator, and the volume adjusted to 2 ml with acetone-alcohol. The remainder of the procedure for total cholesterol was as described by Schoenheimer and Sperry(3).

Results. The data are shown in Table II. The earlier single observation of Swell and Treadwell(1) on the markedly lower hydrolysis rate of a branched chain ester in comparison with the n-acid ester is confirmed and extended. All 8 of the branched chain esters studied were hydrolyzed at a slower rate than the corresponding n-acid esters, and with 2 of the esters the observed hydrolysis was within experimental error of the assay. The data also demonstrate effects of location, degree, and size of branching. Methyl-branches at carbons 2 and 3 of the acids have about the same effect in depressing the hydrolytic activity, while a methyl-branch at carbon 4 has little effect. A second methyl-branch on carbon 2 produces a further depression in activity over that with one methyl-branch. An ethyl-branch at carbon 2 gives a greater depression than a methyl at the same location. In general the closer the branching is to the ester bond and the greater the size of the branch, the greater the depression in hydrolytic activity. These effects are most likely due to spatial interference with the approach of substrate to the active site on the enzyme.

Summary. The synthesis and characteristics of a series of branched chain fatty acid ester of cholesterol are described. The hydrolysis of these esters by pancreatic cholesterol esterase was determined under standard conditions. The branched chain acid esters were hydrolyzed at lower rates than corresponding n-acid esters. The closer the location of the branch to the ester bond, the greater the depression in activity. Esters with ethyl groups on carbon 2 were hydrolyzed at a slower rate than those with methyl groups at this location.

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Total Lipid, Cholesterol, and Phospholipid Content of Non-Irradiated and Irradiated Skin (Rabbits).* (23812)

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Though the lipid composition of the skin may be important for its many functions, the present knowledge of skin lipids is still scanty (1). Only a few species have been examined. Furthermore, the methods which have been available for some comprehensive studies(2) have been inadequate to allow more or less complete characterization of the lipids. The present investigation has been aimed at an examination of the lipids of the rabbit skin with methods developed more recently, as well as at a study of the effect of ionizing irradiation. This paper reports the results of the examination of total lipids, cholesterol, and phospholipids.

Material and methods. We used young adult female albino rabbits about 1700 g weight. Animals were kept on balanced diet *ad libitum* for one week preceding experiments. Hair of both flanks was removed avoiding carefully damage to skin. "Superficial" irradiation was applied at 4000 r or 8000 r (Ritter self rectified machine; Machlett OEG60 Beryllium window tube; 22 cm focal distance; 30 KV; 10 MA; h.v.p.o. 06 mm). Calibration was performed with Victoreen r-meter. One flank of the rabbit was irradiated, the other serving as control, was protected with lead plate. Food was withdrawn following irradiation. Rabbits were

* This work was carried out under contract with U. S. Atomic Energy Commission.

killed by intravenous injection of air 24 hours or in some instances 48 hours after exposure. Immediately after sacrifice, the skin of irradiated and control sides was carefully removed with scalpel so that none of the subcutaneous tissue was included. Occasional histologic checks proved that specimens were free of subcutaneous material. The aliquots for lipid- and dry weight determinations were weighed immediately on a Gram Atic balance. Specimens for lipid examinations were kept frozen with dry ice for the short time required until lipid extraction could be carried out. Method for extraction and purification of lipids used followed the technic of Folch *et al.* (3) with the exception that the very small fluffy, proteolipid layer, which appeared on top of the chloroform phase after washing of lipid extract, was entirely removed. This small fluff, which usually contained less than 20 μg of lipid phosphorus, might otherwise contaminate the extracts sufficiently to cause erroneously high phosphatidylserine figures. The washed lipid extract was evaporated to dryness at 60°C under reduced pressure. The residue was dissolved in hot chloroform:methanol 2:1. The chloroform:methanol extract thus obtained was filtered through a Buchner funnel with a fritted disc of medium porosity. The funnel was washed with hot chloroform:methanol. The filtrate was evaporated again to dryness as described above and the residue was finally extracted with chloroform. This chloroform extract was sufficiently free of nitrogenous impurities. Additional passing of the material through a cellulose column(4) capable of removing even traces of such impurities as amino acids, did not affect the results to be described and was therefore omitted for these determinations. Total lipids were determined by weighing. An aliquot of the chloroform extract was evaporated at room temperature under reduced pressure. The evaporate was dried in vacuum desiccator over P_2O_5 for weight determination. The chemical analyses were carried out with the following procedures: Free- and total cholesterol by the methods of Brand and Sperry(5); total phospholipids by the method of Sperry(6); phospholipids easily hydrolysable with alkali by the method of Schmidt, Tannhauser *et al.*(7); ethanolamine and serine by the method of Axelrod, Reichenthal and Brodie(8); choline by the method of Hack(9); and aldehydes by the method of Feulgen and Gruenberg(10).

Results. The average percent lipid composition of non-radiated and irradiated skin of rabbits based on the dry weight or on the total phospholipid content of the skin are given in Table I and Table II respectively.

Table I shows that cholesterol of the non-

TABLE I. Percent Lipid Composition* of the Non-Irradiated and Irradiated Skin of Rabbits Based on Dry Weight.

	No. of rabbits†	Total	Cholesterol		P-lipids	
			Total	Free	Total	Easily hydrolyzed with alkali
Control	18-33	7.51 ± .93	.43 ± .02	.39 ± .02	1.32 ± .05	1.17 ± .05
Irradiated	18-33	7.89 ± .65	.47 ± .02	.41 ± .02	1.40 ± .06	1.22 ± .06

		"Cephalin"‡			Lecithin
		Total	Ethanolamine containing P-lipids	Serine containing P-lipids	
Control	18-33	.41 ± .016	.28 ± .012	.13 ± .013	.62 ± .045
Irradiated	18-33	.43 ± .026	.29 ± .014	.14 ± .013	.67 ± .048

* Means ± stand. error.

† Only lecithin values were determined in less than 25 rabbits.

‡ The term "cephalin" was used only to allow comparison with previous studies which did not distinguish between ethanolamine or serine containing phospholipids. The terms ethanolamine containing P-lipids or serine containing P-lipids were used instead of phosphatidyl ethanolamine and phosphatidyl serine respectively, because studies not given here have established that these fractions might contain also small amounts, up to 7%, of plasmologens, particularly ethanolamine-plasmologens.

TABLE II. Percent Phospholipid Composition* of Non-Irradiated and Irradiated Skin of Rabbits Based on Total Phospholipids.

		"Cephalin"‡			Lecithin	P-lipids	
	No. of rabbits†	Total	Ethanolamine containing P-lipids	Serine containing P-lipids		Sum of lecithin and cephalin	Easily hydrolyzed with alkali
Control	18-33	34.2 ± 1.3	21.8 ± .6	12.5 ± .8	48.1 ± 2.1	81.6 ± 3.0	87.7 ± 1.6
Irradiated	18-33	31.8 ± 1.6	20.7 ± .7	11.0 ± 1.1	52.9 ± 3.1	78.6 ± 4.0	82.1 ± 4.6

* Mean ± stand. error.
† Only lecithin values were determined in less than 25 rabbits.
‡ Use of terms "cephalin," ethanolamine containing P-lipids and serine containing P-lipids has been explained in Table I.

irradiated or irradiated skin of rabbits comprises mainly free cholesterol. The cholesteryl ester values are not included in the Table, but it can be calculated easily that they amount only to 9.3% or 12.8% of total cholesterol content of non-irradiated or irradiated skin respectively. Both free and total cholesterol values have not been affected significantly by the ionizing irradiation.

Phospholipids of the rabbit skin comprise 3 well defined fractions, *viz.*, lecithin, phosphatidyl ethanolamine and phosphatidyl serine. Occurrence of an additional but rather small plasmalogen fraction, ethanolamine-plasmalogen, has been indicated in Table I. The phospholipid composition of non-irradiated and irradiated rabbit skin based on total phospholipid content is given in Table II.

Table II demonstrates that the phospholipids of non-irradiated or irradiated skin of rabbits alike comprise about 50% lecithin and somewhat over 30% "cephalin." The "cephalin" itself consists of about two-thirds of ethanolamine containing phospholipids (mostly phosphatidyl ethanolamine and small amounts of plasmalogens) and of about one-third of phosphatidyl serine. The sum of all these phospholipid fractions, however, amounts to only about 80% of total phospholipid values. It must thus be assumed that other phosphorus containing compounds must have been present in addition. While no definite opinion about the exact chemical composition of these unidentified lipids can be expressed here, it may be pointed out that only about 82-88% of the phospholipids have been hydrolyzed easily with alkali. Since lecithins and "cephalins" usually can be hydrolyzed in such a way this is an indication that the unidentified lipids may be resistant to alkali-hydrolysis.

Conclusions. 1. Data have been presented on total cholesterol, free cholesterol, phospholipid, and total lipid content of non-irradiated and x-ray irradiated skin of rabbits. 2. Cholesterol of both non-irradiated or irradiated skin alike comprises mostly free cholesterol and only relatively small amounts, 9-13%, cholesteryl esters. 3. Phospholipids of both non-radiated or irradiated skin consists of about 48-53% lecithin, 21% phosphatidyl ethanolamine, 11-12% phosphatidyl serine, rather small amounts of ethanolamine-plasmagens, and a relatively large amount, 18-21%, of unidentified phospholipids. 4. Ionizing irradiation of the skin of rabbits has not significantly affected the lipids under study.

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Host Resistance to Hemorrhagic Shock. XII. Mechanism of Protective Action of Dibenamine.* (23813)

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Protection by dibenamine against the development of irreversibility to transfusion in hemorrhagic shock was first demonstrated by Wiggers *et al.* in dogs(1). This finding was confirmed by Remington *et al.*(2), and later in rats by Shorr *et al.*, who also observed protection against drum shock by dibenamine (3). Jacob *et al.*(4) found that the protective effect of dibenamine given prior to induction of hemorrhagic shock in dogs was difficult to assess, since the hypotensive effect of the drug altered the framework of the standard experiment which they used to produce hemorrhagic shock. The considerably lower bleeding volume in their dibenaminized dogs suggested a less severe degree of shock; for when they were bled a volume equal to that required in untreated dogs to lower the blood pressure to a minimum level (30 mm Hg), they died. This finding was similar to that of Wiggers *et al.*(1), who found that if dibenamine was given to a dog after it had bled out to an arterial pressure of 30 mm Hg, infusion of a considerable fraction of the shed blood was required to prevent a further and lethal decline in arterial pressure. Shorr (personal communication), however, found that dibenaminized rats tolerate as great a bleeding volume as is required in the untreated rat to lower the blood pressure to a minimum level (50 mm Hg).

With the discovery of an endotoxemia in the dog and rabbit in hemorrhagic shock(5), the possible relationship between the protective action of dibenamine and the endotoxin came under consideration. The same question arose when it was shown that dibenamine prevents death from an otherwise fatal type of shock which develops following release of a one hour occlusion of the superior mesenteric artery, and which also appears to be due to endotoxemia(6). With this suggestive evidence that dibenamine might be acting as an anti-endotoxic agent, experiments were undertaken to see if dibenamine in hemorrhagic shock protects by preventing the appearance of the toxin, or by blocking its capacity to damage the peripheral vessels. In this communication evidence is presented that dibenamine protects against the development of irreversibility to transfusion in hemorrhagic shock by blocking the activity of the toxin which kills a non-dibenaminized animal(5).

Method. Adult white rabbits (Av. wt. 2.3 kg) were employed for this study. *Exp. 1. Effect of Dibenamine on the Course of Hemorrhagic Shock.* This experiment was performed in order to see if the protective effect of dibenamine in hemorrhagic shock as observed in dogs and rats is applicable to the rabbit. Hemorrhagic shock was produced as described elsewhere(7). In this experiment the rabbits bleed out into an elevated reservoir so as to lower the blood pressure to 50 mm Hg, where it remains until a transfusion is given. The volume of shed blood in the reservoir reaches a maximum within one hour (Fig. 1), after which it begins to return to the circulation. By or before the 6th hour all or most of the blood has returned spontaneously, and death occurs soon thereafter. If all the blood remaining in the reservoir is rapidly infused at any time after the fourth hour, the pressor response is poor, the rabbit remains prostrate, and death follows in a few hours. All animals show intramural focal hemorrhages in the intestine, a feature which is attributable to the action of endotoxin(5). The effect of dibenamine on the course of these events was determined as follows: In one group of rabbits dibenamine, in a dose of 20 mg/kg, was given intravenously three

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† Holder of Travelling Fellowship from Leeds General Infirmary, Leeds, England.

hours before inducing hemorrhagic shock.‡ In another group twice this dose was given 18 hours before inducing shock in order to avoid the influence of the toxic effects of the drug (*e.g.* convulsive tremors), which develop occasionally shortly after its administration, on the results of the experiment. The shock was continued in these two groups as in non-dibenaminized rabbits for six hours, after which all the shed blood remaining in the elevated reservoir was transfused. The pressor response to transfusion, the presence or absence of the hemorrhagic lesion in the gut,§ and the survival time were noted, and the results compared with those from non-dibenaminized rabbits.

Results. (20 experiments with dibenamine and 15 without dibenamine). The dibenaminized rabbits differed from the controls in many respects.‖ In the dibenaminized rabbits the maximum volume of hemorrhage required to lower the blood pressure to 50 mm Hg, and to keep it at this level, usually was 70% of that in untreated controls which were run simultaneously. The time taken to achieve maximum bleed-out in the dibenaminized rabbits was 2 hours, whereas the maximum bleed-out in the controls occurred within one hour. In the former there was almost complete absence of spontaneous return of blood from the elevated reservoir during the 6 hours of hypotension, and the pressor response to transfusion was significantly greater (72 ± 5 mm Hg) than in the controls (52 ± 4 mm Hg). Restoration to the pre-shock level was nearly complete (–10 mm ± 5 Hg) in the dibenaminized group, as compared to a deficiency of 50 mm ± 5 Hg in the control group. The controls responded poorly to the transfusion, showed the hemorrhagic lesion in the gut wall, and all but one died shortly after transfusion. In spite of a good pressor response the dibenaminized animals continued to be sick, and were unable to drink or eat on return to their cages. All but one developed severe sepsis in the groin wounds and died within 24-48 hours. Postmortem examination disclosed no injury to the gut, which is a characteristic finding in the irreversibly shocked animal.

Comment. The degree of hypovolemia which non-dibenaminized animals display on reaching and remaining at the lowest level of blood pressure they can tolerate for several hours (50 mm Hg in the rabbit, 40-45 mm Hg in the rat, and 30 mm Hg in the dog) is a constant (± 52 ± 10 ml). Hence the severity of hemorrhagic shock in all 3 species is a function of the degree of hypovolemia as well as the degree of hypotension. Since it is not possible in most experiments in the rabbit, rat or dog(1,2,4) to produce conditions with and without dibenamine that are comparable in terms of these 2 major dependent variables, one cannot conclude from the results obtained that dibenamine protects the animal. If the results of the experiments with and without the drug are compared in terms of degree and duration of hypotension, protection appears to have been obtained. But if the experiments are performed so as to produce the same degree of hypovolemia, no protection is obtained. Thus, 6 non-dibenaminized rabbits were bled to 50 mm Hg, and immediately after reaching the maximum bleed out they were equated with dibenaminized rabbits in terms of degree of hypovolemia by returning 30% of the maximum bled volume. The blood pressure rose to 80-88 mm Hg and remained there for 6 hours, with prompt and sustained recovery in response to transfusion at that time. The foregoing experiments, therefore, leave the issue in doubt. A better judgment as to whether dibenamine protects might be reached by observing whether this drug does or does not prevent the endotoxemia of hemorrhagic shock, which accounts for the

‡ Preliminary studies confirmed in rabbits the finding of Ahlquist in the dog(8) that dibenamine in a dose of 20 mg/kg body weight produces adrenergic blockade within 15 minutes. Maximum blockade was established in 25 min. The pressor action of epinephrine was never completely blocked, for 0.2 ml of a 1/10,000 solution still produced a small initial pressor response, followed by the characteristic reversal. Duration of reversal was longer, the longer the interval between administration of dibenamine and injection of epinephrine.

§ This lesion was looked for in survivors, which were killed at 48 hours, as well as in those that died.

‖ There was no difference between the results of the 2 groups of dibenaminized rabbits.

development of irreversibility to transfusion. To this end Experiment 2 was performed.

Exp. 2. Effect of Dibenamine on Toxicity of Blood in Hemorrhagic Shock. In this experiment 30 rabbits were put into hemorrhagic shock as described above. One group of 15 rabbits received no dibenamine. The other group of 15 rabbits was given dibenamine (20 mg/kg) 3 hours before inducing shock. All 30 rabbits were exsanguinated after 6 hours of shock. Each blood was tested for toxicity by a method described previously(5). All blood from non-dibenaminized animals displayed the presence of toxin. Of the 15 bloods from dibenaminized animals 11 were free of toxin, and 4 were toxic. It is noteworthy, however, that the degree of hypovolemia was less than in the controls in all 11 dibenaminized rabbits with non-toxic blood, and the same as in the controls in all 4 dibenaminized rabbits with toxic blood.

Comment. These results are not more definitive than those of Exp. 1; for it is as valid to conclude that the toxicity of the blood is governed by the severity of the shock (degree of hypovolemia) as it is that dibenamine is acting directly to prevent toxin. To determine more definitively whether dibenamine acts directly to prevent toxicity it is necessary to test the action of dibenamine when the hypotension and hypovolemia are the same as in the non-dibenaminized animal. This was done in Exp. 3.

Exp. 3. Effect of Dibenamine (20 mg/kg) on Vulnerability of Rabbits in Reversible Shock to Toxic Blood. In this experiment 10 rabbits were bled to a blood pressure of 50 mm Hg and maintained at this pressure for 1½ hours, at which time they were transfused with toxic blood from rabbits dying of shock of 6 hours duration. Dibenamine (20 mg/kg) was given I.V. in 10 cc of saline 15 minutes before the transfusion. As already reported, non-dibenaminized rabbits transfused after 1½ hours of shock with toxic blood lie prostrate after the transfusion, and 90% die within 18 hours(5). The results of the current experiments on rabbits given dibenamine were as follows: Six rabbits recovered completely and 4 died. Of the 4 which died, one died after 24 hours with the hemorrhagic lesion in the gut wall. The other three died after 3 or 4 days with wound sepsis, and without hemorrhage in the gut wall.

Comment. The saline given with the dibenamine was of no therapeutic importance, for in previous experiments many non-dibenaminized rabbits receiving as much or more saline at the time of the transfusion with toxic blood did not survive. Whether dibenamine was already exerting an anti-adrenergic effect at the time of the transfusion was not certain. In seven of the 10 experiments the blood pressure did not fall after it was given, but the pressor response to transfusion was lower (92 mm Hg mean systolic blood pressure) than in 5 control experiments (98 mm Hg mean systolic pressure).¶ In any case, dibenamine appears to have acted to prevent injury to the peripheral vessels by the injected toxin.

Discussion. How does dibenamine block the action of toxin? There is strong evidence that this toxin is an endotoxin(9,10). Thomas (11) has shown that endotoxin produces injury to the peripheral vessels with hemorrhagic extravasation and necrosis by an effect upon epinephrine. Hence the protective effect of dibenamine in hemorrhagic shock, *i.e.* blockade of the effect of the endotoxin upon the peripheral vessels and prevention of the hemorrhagic lesion in the gut, appears to be attributable to its antiadrenergic property. But dibenamine may also protect in virtue of its action on other tissues which govern the peripheral circulation, *e.g.* those controlling the release of serotonin(12), and which, in its absence, might be damaged by endotoxin. Whatever its sites of action may be, dibenamine preserves peripheral flow in shock(13). Thus the integrity of the tissues concerned with the detoxification of endotoxin may be sustained, and the responsiveness of the peripheral circulation to transfusion preserved.

Summary and conclusions. Since the development of irreversibility to transfusion in hemorrhagic shock has been shown to be

¶ These control experiments were performed for the specific purpose of comparing the pressor response with and without dibenamine. The volume of the transfusion was about the same in the controls as in the dibenaminized rabbits (65 ± 2 ml).

caused by bacterial endotoxins, and dibenamine prevents the development of irreversibility, the blood of dibenaminized rabbits in hemorrhagic shock was tested for the presence of endotoxin and found to be toxin free. But because the blood loss in the dibenaminized animal in shock is less than in the non-dibenaminized animal, the absence of toxin might be due to the lesser hypovolemia rather than to the dibenamine. Accordingly, an additional experiment was performed to see if dibenamine given to the rabbit in reversible shock will permit it to survive a transfusion of toxic blood from an irreversibly shocked donor—a procedure which kills the non-dibenaminized rabbit in reversible shock. This experiment demonstrates that dibenamine blocks the action of the toxin, and thereby not only preserves the responsiveness of the circulation, but also prevents the hemorrhagic lesion in the bowel wall, which is characteristic of irreversible shock.

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Circulatory Reactions of Dogs and Rats to Polyvinylpyrrolidone or Dextran After 48/80. (23814)

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Dogs and rats injected with the potent histamine liberator 48/80 become temporarily refractory to further administration of the same dose(1-2). Temporary tachyphylaxis also occurs in dogs after repeated injections of polyvinylpyrrolidone (PVP) and in rats after dextran(3). These colloids act as histamine releasers in each species respectively(4-5). We have sought evidence of cross-refractoriness using two criteria of sensitivity. One is the appearance of bluing in animals which have been injected with Evans blue (T-1824), and indicates an increase in skin capillary permeability to the albumin-dye complex. The other is a decrease in mean arterial blood pressure, indicative of a systemic reaction.

Methods and results. Details of the experimental procedure and summary of the results appear in Table I. No experiments are included in which the blood pressure at time of challenge was not 90 mm Hg or higher. Evans blue was given usually early in the experiment and in dosages (0.6-2 mg/kg) which produce no cutaneous bluing in untreated animals. In the first experiment 6 dogs received intravenously a series of graded doses of 48/80* as used by Slomka and Goth(6). After 44-112 μg/kg, small foci of blue became visible, surrounded by erythema. Gradually the bluing extended further and the flush faded. Ten minutes after a total of about 600-700 μg/kg, the mean blood pressures measured by catheter in a femoral artery were 20-50 mm

* Available through the kindness of Dr. E. J. de Beer, Wellcome Research Laboratories.

TABLE I. Circulatory Reaction of Dogs and Rats to 48/80 and PVP or Dextran.

No.	Pretreatment (per kg body wt)	No. reacting to last inj.		Challenging treatment (per kg body wt)	No. reacting to challenge	
		Vasodepression*	Bluing		Vasodepression*	Bluing
	Dogs conscious or anesthetized with sodium thiopental					
6	48/80, 800-900 μg in 14-17 doses	0	0†	PVP, 35 mg	6	6
6	PVP, 35-70 mg in 2 doses	0	0†	48/80, 100 μg	6	1
6	None			*Idem*	4	2
	Rats anesthetized with sodium pentobarbital					
10	48/80, 600-1300 μg in 4-9 doses	0	1‡	Dextran, 600 mg	0	2
10	Dextran, 1200 mg in 2 doses	0	1‡	48/80, 200-300 μg	2	0
16	None			48/80, 200 μg	13	2

* Decrease in mean arterial pressure of 25 mm Hg or more. † Bluing had appeared in all animals before last inj. ‡ Bluing had appeared in a few animals before last inj.

Hg below their preinjection values. At this time a rapid injection of 200 μg/kg 48/80 produced no further change in pressure or color, showing the animals to be refractory to this dosage. About an hour later (in one animal only 10 minutes later), when the mean pressures were normal, PVP† was injected and the usual vasodepression followed (Fig. 1A). Bluing became more generalized and edema more intense.

Six dogs were shown to be non-reactive to a 2nd PVP injection 1 hour after the first. They were challenged with 100 μg/kg 48/80 and the blood pressures fell 25-60 mm Hg (Fig. 1B). In the first 3 dogs no additional bluing appeared. In the last 3, dye was given just before 48/80 so that an adequate concentration in the circulation was assured, which may not have been the case in the first 3 dogs injected with dye before PVP. A trace of bluing appeared in one. The results in this group resemble in general those seen in 6 additional dogs given 100 μg/kg without pretreatment.

For the experiments with dextran adult male and female rats of the Sprague-Dawley or Osborne-Mendel strains were anesthetized with sodium pentobarbital and the usual dose of Evans blue was administered. Pressures were recorded from the common carotid artery. Ten rats received intravenous injections of graded doses of 48/80 until they were refractory to 200 μg/kg. Dextran‡ was then administered, and the vasodepression usual in this species did not occur (Fig. 2A) and additional bluing was seen in only 2 animals.

Ten rats that were non-reactive to a 2nd dextran injection were challenged with 48/80. Only 2 had a significant fall in blood pressure (25 and 35 mm Hg respectively) and none of them had additional bluing (Fig. 2B).

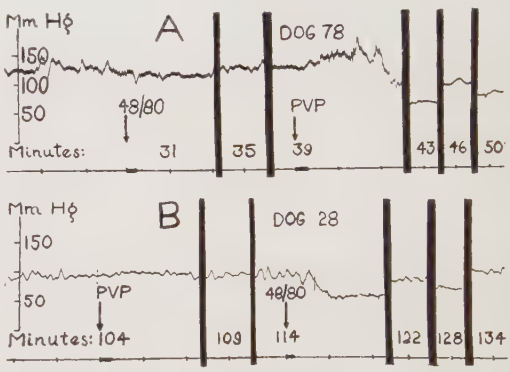

FIG. 1. Mean arterial blood pressure records from 2 unanesthetized dogs. A: dog 78, 12/7/56. Fourth inj. of 48/80 (200 μg/kg) at 30 min. PVP (35 mg/kg) at 39 min. was followed by vasodepression and bluing typical of an initial PVP administration. B: dog 28, 12/6/57. Second inj. of PVP (35 mg/kg) at 104 min. 48/80 (100 μg/kg) at 114 min. was followed by vasodepression and erythema but no bluing.

† PVP-Macrose (3.5% PVP in isotonic salt solution), kindly furnished by Schenley Laboratories.

‡ "Expandex", 6% dextran in isotonic NaCl.

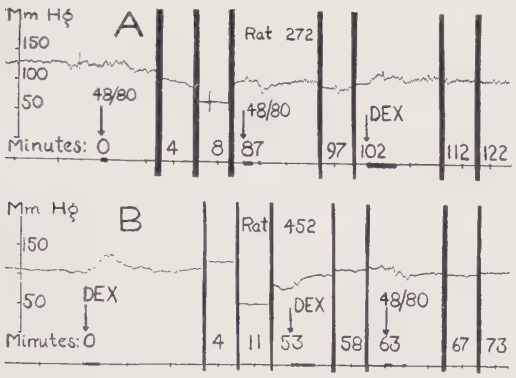

FIG. 2. Mean arterial blood pressure records from 2 anesthetized rats (sodium pentobarbital, about 35 mg/kg i.p.). A: rat 272, 12/19/56. First inj. of 48/80 (200 μg/kg) at 0 min., last inj. at 87 min. (300 μg/kg; total dosage 900 μg/kg). At 98 min. blue seen between toes. At 102 min. dextran (600 mg/kg) was inj., followed by edema and bluing but no fall in blood pressure. B: rat 452, 8/16/57. At 0 min. dextran (600 mg/kg) inj. was followed by the usual prolonged vasodepression and moderate bluing. At 53 min. a similar inj. raised the pressure. At 63 min. 48/80 (200 μg/kg) produced little effect. Compare with A at 0 min.

This is in contrast to vasodepression appearing in 13 out of 16 previously untreated rats.

Discussion. Our finding that dogs refractory to 48/80 still respond to PVP corroborates results with another histamine liberator, Tween 20(6) and substantiates Halpern's statement(7). On the other hand, cross-refractoriness to other histamine liberators has been demonstrated in this species(8-9). Slomka and Goth(6) produced refractoriness in dogs to 48/80 as shown by absence of vasodepression and without increased circulating histamine, but do not mention evidence of local permeability changes. The consistent appearance of bluing in our dogs given large doses of 48/80 is evidence that histamine or some other permeability affective substance is present.

The reaction to 48/80 of rats anesthetized with a barbiturate resembles that after dextran. The absence of bluing may be caused by low hydrostatic pressure precluding the passage of dye into tissue spaces or by failure of flow through closed capillaries. Ether has been shown(10) to protect rats from dextran vasodepression as does 48/80, but there is the difference that with ether the incidence of bluing is high. In the experiments of Feldberg and Talesnik(11) rats after extensive 48/80 administration had an attenuated or negative response to egg white. Halpern(7) states that rats depleted of histamine by dextran react to 48/80, which is the opposite of our conclusions using the criteria described.

Separate consideration of the two events, vasodepression and bluing, permits more precise conclusions regarding cross-refractoriness, and hence, mechanisms. The two phenomena may be due to histamine released either at different sites or in different amounts at the same site, or to the presence of some additional substance, such as 5-hydroxytryptamine(12).

Summary. The appearance of cutaneous bluing and vasodepression has been followed after T-1824 and 48/80 injection in dogs and rats subsequently challenged with polyvinylpyrrolidone (PVP) and dextran respectively. The reverse sequence of injection was also tested. Graded doses of 48/80 in dogs resulted in refractoriness to the vasodepressive effects of similar doses, but cutaneous bluing was always seen. This increased skin capillary permeability may be interpreted as evidence of local release of histamine or other substance. In dogs it was confirmed that there was no cross protection between PVP and 48/80. In rats anesthetized with a barbiturate and pretreated with 48/80 the usual vasodepression and bluing after dextran did not occur. Rats refractory to dextran were also refractory to 48/80.

We are indebted to George E. Clipper for capable technical assistance.

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Selenium and Development of Exudative Diathesis in Chicks.* (23815)

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A number of workers have reported the development of exudative diathesis as a symptom of Vit. E deficiency using the diet of Scott *et al.*(1). It was recently reported that organic complex of selenium was the factor 3 of dried brewers yeast necessary for prevention of liver necrosis in rats fed a similar Vit. E-free Torula yeast diet(2); and selenium was also found to be active in this regard. Schwarz *et al.*(2) have shown that selenium or factor 3 concentrates would prevent the gross symptoms of exudative diathesis in chicks fed Torula yeast. Patterson *et al.*(3) have identified selenium as the active component of casein and defatted pork kidney in preventing development of exudative diathesis in chicks. Previous work has shown that exudative diathesis of the chick or poult resulted in marked diminution of hemoglobin level, total serum protein (albumin level), and total red cell counts. These conditions could be prevented by adding Vit. E or substituting 10% dried brewers yeast in the Torula yeast diet(4,5).

In view of these results it seemed desirable to determine the effect of selenium on occurrence of the blood changes associated with exudative diathesis.

Methods. In the first study, 5 groups of 20 White Rock chicks were placed on the Torula yeast diets supplemented as shown in Table I. The basal diet used in this study is the same as that of Creech *et al.*(5). Selenium was supplied as sodium selenate at levels of 1 and 10 ppm. At the end of 3 weeks, when gross symptoms of exudative diathesis had appeared in birds fed the basal diet, a representative sample (5 birds per group) was selected for the determination of total serum protein, albumin:globulin ratios, red cell counts, hemoglobin, and packed cell volume as previously described(5). The second study in this series employed 20 New Hampshire chicks per group with the basal diet supplemented as shown in Table III. Selenium was supplied as sodium selenate (1, 0.5, 0.1 ppm), as the selenite (1 ppm), and as free selenium (0.5 ppm). At 3 weeks of age a representative sample of birds from each group was removed for the blood analyses previously listed. The birds in both experiments were housed in electrically heated batteries with raised wire floors; feed and water were supplied *ad libitum*. Body weights were determined at weekly intervals throughout the study, and daily observations were made for the appearance of symptoms of exudative diathesis.

Results. The addition of selenium as the selenate either at the level of 1 ppm or 10 ppm completely prevented development of gross symptoms of exudative diathesis up to

* This work was supported in part by grants-in-aid from Robert A. Welch Fn., Brewers Yeast Council, and Distillation Products Industries. Torula yeast was supplied by Lake States Yeast Corp., vit. E and vit. E-free lard by Distillation Products Industries; biotin by Hoffmann-La Roche; methionine and glycine by Dow Chemical Co.; stabilized vit. A by Stabilized Vitamins; chlortetracycline and folic acid, by Lederle Laboratories; menadione sodium bisulfite by Abbott Laboratories; vit. D_3 by Charles Bowman and Co.; and other B vitamins by Merck and Co.

4 weeks of age, as previously reported by Schwarz *et al.*(2). Symptoms of exudative diathesis were likewise prevented by addition of 20 mg of *d*-alpha tocopheryl acetate per lb or substitution of 10% dried brewers yeast in the Torula yeast basal diet (Table I). The first symptoms of exudative diathesis appeared in the basal group on the 17th day and increased to an incidence of 65% by the 21st day. Birds fed 1 ppm selenium or 10 ppm selenium appeared to be depressed in growth when compared to the birds fed 20 mg of Vit. E/lb or 10% dried brewers yeast (Table I).

The serum albumin:globulin (A/G) ratios, as determined by electrophoresis at 21 days, were markedly reduced in birds receiving the unsupplemented Torula yeast basal diet (Table I). The A/G ratio of this group was 0.16 whereas the birds receiving 20 mg of *d*-alpha tocopheryl acetate per lb of feed exhibited an A/G ratio of 0.73, and the dried brewers yeast fed group had a ratio of 0.80. A partial improvement in the A/G ratio was observed with the addition of selenium to the basal diet. The 1 ppm level (selenate) resulted in an A/G ratio of 0.5, while the 10 ppm level resulted in a ratio of only 0.32. The latter may have been due to toxicity of the higher level of selenium.

A marked reduction in the number of red blood cells was noted in the unsupplemented group (1.8 million). This level was elevated to 2.12 million in the Vit. E supplemented group and to 2.32 million in the dried brewers yeast fed group (Table II). The 1 ppm level of selenium resulted in a cell count of 2.05 million, which is approximately normal under the conditions of this study, and the 10 ppm level of selenium produced a 1.92 million cell count. Hemoglobin levels were markedly reduced in the basal diet (5.11) and returned to normal with the feeding of Vit. E or dried brewers yeast. Very little improvement was observed after addition of selenium to the basal diet. The mean cell volumes were affected only to a slight extent by any of the supplements, or the deficiency of Vit. E in this study. The total serum protein level was increased from 3.18 in the unsupplemented group to 3.77 in the group fed 20 mg of Vit. E per lb of diet, and to 4.47 in the group fed 10% dried brewers yeast. Addition of 1 ppm of selenium resulted in a total protein level, as determined chemically, of 3.47 which represents a slight increase over that obtained with the unsupplemented diet. The 10 ppm level of selenium failed to produce an increase of total serum protein level.

In the second study (Table III), it was found that as little as 0.1 ppm of selenium, added as sodium selenate, resulted in complete protection from symptoms of exudative diathesis to 4 weeks of age. One ppm of selenium as selenite and dried brewers yeast at a 10% level exerted a protective effect with regard to exudative diathesis. However, when the ash of dried brewers yeast was fed at the level equivalent to 10%, a 95% incidence of exudative diathesis was noted by the end of the 4th week. The brewers yeast was burned over an open flame until charring had been initiated and then placed in a muffled furnace at 400° to 500°C for 12 hours. Under these conditions it might be expected that selenium would be lost to a significant extent from the dried brewers yeast ash. The addition of 0.5

TABLE I. Effect of Selenium on Growth and Incidence of Exudative Diathesis in Chicks Fed a Torula Yeast Diet.

Supplement to basal diet	Avg wt 21 days	Avg wt 28 days	% birds showing symptoms	A/G ratio, 21 days
None	240	260 (3 birds)	100*	.16
20 mg vit. E/lb	220	276	0	.73
10% dried brewers yeast	240	309	0	.80
1 ppm selenium (Selenate)	213	255	0	.50
10 ppm *Idem*	187	223	0	.32

* First developed on 17th day; 65% incidence by 21st day.

TABLE II. Effect of Selenium on Blood Picture of Torula Yeast Fed Chicks at 21 Days.

Supplement to basal diet	R.B.C. in millions	Hemoglobin, g %	Mean cell vol, mm^3 (10^{-7})	Total serum protein
None	1.80	5.11	156	3.18
20 mg vit. E/lb	2.12	6.69	142	3.77
10% dried brewers yeast	2.32	7.11	143	4.47
1 ppm selenium (Selenate)	2.05	5.35	134	3.47
10 ppm *Idem*	1.92	5.33	141	3.04

TABLE III. Effect of Selenium on Growth and Incidence of Exudative Diathesis in Chicks Fed a Torula Yeast Diet.

Supplement to basal diet	Avg wt		% exudative diathesis		Appearance of symptoms, days	% mortality at 28 days
	3 wk	4 wk	3 wk	4 wk		
None	190	247 (7 birds)	76	95	17	65*
20 mg *d*-alpha tocopheryl acetate	220	312	0	0		
1 ppm Se (sodium selenate)	166	204	0	0		5†
.5 ppm *idem*	200	293	0	0		
.1 ppm "	237	337	0	0		
1 ppm Se (sodium selenite)	212	297	0	0		
10% dried brewers yeast	224	323	0	0		
Dried brewers yeast ash (equivalent to 10% dried brewers yeast)	192	225 (2 ")	35	95	19	90
.5 ppm Se (free selenium)	182	167 (4 ")	35	80	19	80

* Two birds died at 8th day without symptoms.
† One out of 20 birds died on 21st day without symptoms.

ppm selenium as the free metal failed to produce complete protection against exudative diathesis since there was an 80% incidence of this condition at the end of the 4th week. The growth rate of the birds to 4 weeks of age appeared to be increased by feeding 0.1 ppm selenium as selenate. The average 4-week weight of these birds was 337 g as compared to 312 g for the Vit. E supplemented group. Only 7 birds remained in the unsupplemented group at the end of the 4th week with an average weight of 247 g. In this study, 1 ppm selenium again seemed to produce a depression in growth rate.

Red blood cell counts again were reduced in the absence of Vit. E, to 1.93 million, whereas the Vit. E supplemented group exhibited a count of 2.52 million, (Table IV). The 3 levels of selenium supplied as the selenate (1 ppm, 0.5 ppm and 0.1 ppm) produced counts of 2.12, 2.37, and 2.47 million, respectively. Addition of 1 ppm selenium as selenite produced a count of 2.26 million. Dried brewers yeast again resulted in an elevation of the counts in the birds fed this diet. The ash of dried brewers yeast failed to produce an appreciable effect on the counts. A 0.5 ppm level of free selenium seemed to exert some influence with regard to counts.

Hemoglobin levels were elevated with addition of Vit. E, selenium, as a selenate at a level as low as 0.1 ppm, as the selenite at a level of 1 ppm, and 10% dried brewers yeast. No significant improvement was obtained with the dried brewers yeast ash, under the conditions of this study, however, 0.5 ppm of free selenium did seem to exert some influence with regard to hemoglobin level. It should be noted that no evidence has been obtained that selenium, Vit. E, or dried brewers

TABLE IV. Effect of Selenium on R.B.C., Hemoglobin, Mean Cell Volume and Total Serum Protein in Chicks on Torula Yeast Diet.

Supplement to basal diet	R.B.C. in millions	Hemoglobin, g %	Mean cell vol, mm^3 (10^{-7})	Total serum protein (chemical)	A/G electrophoresis
None	1.93	4.23	145	3.2	.13
20 mg *d*-alpha tocopheryl acetate	2.52	6.86	125	4.0	.42
1 ppm Se (sodium selenate)	2.12	6.37	120	3.8	.30
.5 ppm *idem*	2.37	5.27	126	3.9	.33
.1 ppm "	2.46	6.71	133	4.2	.42
1 ppm Se (sodium selenite)	2.26	6.36	145	4.0	.43
10% dried brewers yeast	2.87	6.76	116	4.4	.58
Dried brewers yeast ash (equivalent to 10% dried brewers yeast)	1.81	4.52	100	3.5	.29
.5 ppm Se (free selenium)	2.48	5.88	124	3.4	.21

yeast exert any effect pertaining to hemoglobin formation other than protection of the birds from the hemorrhaging which occurs in exudative diathesis.

Total serum protein levels were elevated by addition of Vit. E, selenium, as the selenate at all 3 of the levels tested, as the selenite, and by 10% dried brewers yeast (Table IV). No increase was noted after the feeding of dried brewers yeast ash or selenium as the free metal. A/G ratios, as determined by electrophoresis, were again increased with Vit. E, the 3 levels of selenate added to the diet, the selenite and 10% dried brewers yeast. Only a slight improvement with regard to A/G ratio was noted upon feeding 10% dried brewers yeast ash, as was also true with the addition of 0.5 ppm selenium. Selenium, as selenate, at a level of 1 ppm was apparently not as effective and perhaps was detrimental as compared to the level of 0.1 ppm. The A/G ratio obtained with 1 ppm selenium from selenate was 0.30. The A/G ratio obtained with 0.1 ppm was 0.42 which was identical to that obtained with 20 mg *d*-alpha tocopheryl acetate per lb of feed. The feeding of 10% dried brewers yeast resulted in an A/G ratio of 0.58 which was the highest obtained in this study.

The results of these studies show that addition of selenium to the Torula yeast basal diet will result in protection of the birds against symptoms of exudative diathesis, as reported by Scott(1). Free selenium appears to be only partially effective in overcoming the symptoms. The addition of a smaller quantity of selenium as selenate (0.1 ppm) will produce changes, with regard to red blood cell counts, hemoglobin, total serum protein, and A/G ratios, identical to those obtained by the feeding of 20 mg of *d-alpha* tocopheryl acetate per lb of Torula yeast basal diet. These data indicate that 1 ppm of selenium as selenate was toxic and therefore, even though protection was obtained from the gross symptoms of exudative diathesis, the blood components studied were adversely affected as a result of the toxicity of the selenium rather than by a Vit. E deficiency.

Summary. Two experiments have been conducted with White Rock and New Hampshire chicks concerning effects of selenium, Vit. E and dried brewers yeast on occurrence of exudative diathesis, and of these substances on blood changes associated with Vit. E deficiency. Selenium (selenate) at 0.1 ppm was effective in reversing the lowered red blood cell counts, lowered hemoglobin values, lowered serum protein levels, and the reduced A/G ratios associated with exudative diathesis. As this level was increased to 1 ppm the total blood changes were not as greatly affected as with feeding of 0.1 ppm level, apparently due to the toxicity of selenium.

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Blood and Urine Tyrosine Levels in Starved X-irradiated Rats. (23816)

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Davy(1) reported that dogs exposed to a 500 r dose of x-rays showed immediate fall in serum albumin with return to normal after 48 hours. Muntz, Barron and Prosser(2) indicated that a lethal total body dose of x-rays produced, in dogs, no characteristic changes in plasma protein pattern after one week following radiation. Thereafter, until death of the animal the serum proteins diminished steadily. Similar decreases in serum proteins

TABLE I. Serum Tyrosine Levels in 800 r Total Body X-Irradiated Starved Rats.

Time following radiation exposure (hr)			No. of rats	Mean serum tyrosine level, μg tyrosine equivalent / ml of serum	P level
24	starved	irrad.	7	54.4 ± 8.8	.019
	"	control	10	44.8 ± 4.9	
48	"	irrad.	9	61.2 ± 9.6	.007
	"	control	7	77.6 ± 9.6	
72	"	irrad.	10	45.1 ± 8.2	>.050
	"	control	8	53.2 ±11.2	
96	"	irrad.	5	35.0 ± 3.7	"
	"	control	10	35.4 ± 7.2	
Normal		control	15	52.4 ± 8.8	

were also reported by Goldwater and Entenman(3). Other work indicates variations in the amino acid excretion patterns in animals treated with ionizing radiations, Hallesy and Doull(4,5), Mefferd and Martens(6). These variations in blood and urine amino acid levels reflect protein changes resulting from radiation exposure. Herriott(7) found that as protein hydrolysis progressed the intensity of the blue color increased when the Folin-Ciocalteau phenol reagent was reacted with the supernatant from an active protein-enzyme system. In this report Herriott's method is used exclusively in following the protein changes, in terms of tyrosine, in the blood and urine of starved control rats and of starved x-irradiated rats.

Materials and methods. Adult male Sprague-Dawley rats weighing between 200 and 250 g were housed in a temperature controlled room maintained at about 75°F. Water was provided *ad libitum,* but food was withheld during the post-irradiation period. X-irradiation was administered as a single total body dose of 800 r from a General Electric Maxitron Unit operated at 250 kv and 30 ma. Added filtration consisted of 1 mm Al plus 0.5 Cu, HVL 1.1 mm Cu, with the tube to target distance measuring 50 cm. The dose rate was 122 r/min as measured in air with a Victoreen 250 r Ionization Chamber. Blood was obtained by cardiac puncture under nembutal anesthesia, allowed to clot and aliquots of separated serum were subjected to quantitative analysis for tyrosine(7). Urine was collected daily, the 24 hours total output noted and aliquots were analyzed for tyrosine content.

Results. Table I shows mean serum tyrosine levels in starved rats at various intervals following 800 r total body x-irradiation. Results are for samples collected at 24, 48, 72 and 96 hours post-irradiation for starved irradiated rats and for starved control rats. In all cases, serum tyrosine level was expressed as μg tyrosine equivalent/ml of serum. At 24 hours post-irradiation, the serum tyrosine level was greater for starved control rats than for starved irradiated rats. At 48 hours the level in starved irradiated rats increased; whereas, an appreciable increase was noted for starved control rats. For 72 and 96 hours post-irradiation a continued decrease in the level occurred for both groups, dropping below the level of normal control rats. Starved irradiated and starved control serum levels were significantly different only for the 24 and 48 hour intervals.

The mean urine tyrosine levels in starved irradiated rats at various intervals following 800 r total body x-irradiation are shown in Table II. The level for starved irradiated rats was higher at all time intervals post-irradiation than for starved control rats. The difference between mean levels for these groups was statistically significant at all time intervals following irradiation exposure.

The data indicate that alterations occur in protein metabolism when food intake is restricted. It appears that protein catabolism

TABLE II. Urine Tyrosine Levels in 800 r Total Body X-Irradiated Starved Rats.

Time following radiation exposure (hr)			No. of samples	Mean urine tyrosine level, μg tyrosine equivalent / day • g body wt	P level
24	starved	irrad.	25	32.9 ± 3.4	<.001
	"	control	30	23.1 ± 4.4	
48	"	irrad.	30	19.1 ± 5.4	.024
	"	control	20	15.9 ± 4.2	
72	"	irrad.	20	16.7 ± 5.5	<.001
	"	control	20	11.4 ± 2.1	
96	"	irrad.	6	15.4 ± 4.8	.004
	"	control	10	9.4 ± 2.8	
Normal		control	18	61.5 ±10.0	

is somewhat slower following irradiation at the level used in this study.

Summary. Alterations in serum and urine tyrosine levels were observed in starved irradiated rats and in starved control rats. It appears that protein catabolism is somewhat slower following irradiation at the 800 r level we used.

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Influence of Oxidation Products of Epinephrine upon Adenosinetriphosphatase Activity of Uterine Muscle Preparation.* (23817)

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The physiological response of uterine muscle to epinephrine has been extensively studied, but biochemical aspects of the epinephrine effect have not been elucidated. The typical response of the non-pregnant uterus to epinephrine in most species is diphasic with an initial contractile response followed by a longer period of inhibition(1,2). The diphasic response is not typical during pregnancy(1). An attempt has been made to gain information concerning the biochemical changes accompanying hormonal stimulation of uterine contractility. It was found that the dephosphorylation of adenosinetriphosphate (ATP) by a contractile protein preparation from bovine uterine muscle was inhibited by oxidation products of epinephrine. Although the enzymatic activity is referred to here as adenosinetriphosphatase (ATPase) activity, it is probable that more than one enzyme is involved.

Materials and methods. The procedure commonly used for preparation of structural elements from skeletal muscle(3,4) was modified for isolation of the contractile elements of uterine muscle. Uterine tissue was obtained from 4 non-pregnant cows which, from examination of ovaries and luminal fluids, were apparently not in estrus at time of slaughter. The endometrium was carefully separated and discarded. The myometrium from the 4 animals was pooled and then chopped in a chilled meat grinder. The extraction and purification were carried out at 5°C. Glass-distilled water was used. One hundred and five grams of tissue were extracted with 350 ml of a solution 0.3 *M* in KCl, 0.15 *M* in K_2HPO_4 and 0.15 *M* in KH_2PO_4 by constant agitation with an electric stirrer for 5½ hours. The mixture was diluted with 1.4 liters of water and filtered through cheese cloth. The filtrate was diluted with water to 4.2 liters. Precipitation of protein was complete in about 3 days. Most of the supernatant was siphoned off and the rest removed by centrifugation. The precipitate was dissolved in 2 *M* KCl. The solution was then diluted to KCl concentration of 0.44 *M* and centrifuged to remove any precipitated material. The supernatant was filtered through glass wool, and diluted with water to KCl concentration of 0.22 *M*, which resulted in precipitation of the desired material. Pre-

* This work was initiated while the senior author was a graduate student in Department of Dairy Science at University of Illinois and has been continued under the auspices of the U. S. Atomic Energy Commission.

cipitate was collected by centrifugation and dissolved in 2 *M* KCl. Purification procedure was repeated 3 more times. Within the range of ultraviolet light from 260 mμ to 290 mμ, this preparation showed a maximum absorption at 260 mμ with a 260 mμ/280 mμ ratio of 1.34. This ratio is explained by the presence of nucleic acid(6,7). The nature of the constituent proteins and nucleoproteins is discussed in more detail by Snellman and Tenow (5), who studied preparations similar to the one used here. *ATPase* was assayed at 38°C by measurement of rate of inorganic phosphate appearance under anaerobic conditions in $NaHCO_3$ buffer at pH 7.3 in the presence of added ATP. Warburg flasks were used with a gas phase of 95% N_2, 5% CO_2 mixture. Results represent calculations upon total phosphate released enzymatically. Inorganic phosphate was determined by the method of Fiske and SubbaRow(8); 4% trichloroacetic acid was used as protein precipitant. *Oxidation product* or products of epinephrine were estimated as adrenochrome. Measurements of optical density of reaction mixtures in one group of experiments and of partially oxidized epinephrine solutions in a second group were used to estimate concentration of oxidation products of epinephrine. Measurements made using the Klett-Summerson photoelectric colorimeter with No. 50 filter were compared to a standard adrenochrome assay(9). No further attempt was made to identify the oxidation products for these experiments.

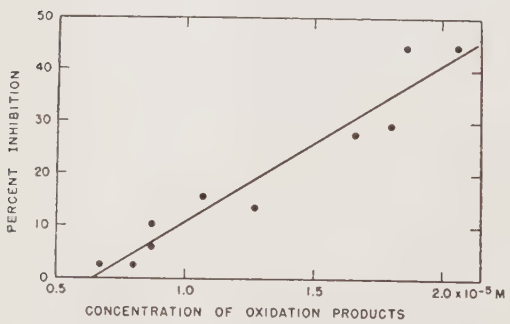

FIG. 1. Relationship between molar concentration of oxidation products of epinephrine, estimated as adrenochrome, and inhibition of ATPase activity. Reaction mixtures contained 37.5 μg N/ml, 0.0260 *M* $NaHCO_3$, 0.5 *M* KCl, and 0.005 *M* ATP. Regression and associated stand. error for relationship between percent inhibition of ATPase activity and concentration of oxidation products was 30.26 ± 2.89. Stand. dev. from regression was 4.43. Regression was highly significant ($p = < 0.001$).

Results. In the first group of experiments the uterine preparation was incubated with epinephrine (6×10^{-5} *M*) under aerobic conditions for periods of time ranging from ¼ to 30 minutes before gassing with 95% N_2-5% CO_2. As aerobic incubation period increased there was a progressive increase in epinephrine oxidation. After 20 minutes of anaerobic gassing the assay was begun by addition of ATP from the side arm. Estimations of adrenochrome concentration were made at the end of the assay period. The average ATPase of the controls was 0.70 μM of phosphate liberated from ATP per ml reaction mixture (37.5 μg N) per hour. Fig. 1 presents the relationship found between oxidation products of epinephrine and inhibition of ATPase activity.

In another group of experiments, solutions of epinephrine were allowed to autoxidize in bicarbonate buffer; oxidation was terminated by anaerobic gassing. Adrenochrome concentration was determined immediately prior to use. The solutions were kept anaerobic until they were used; aliquots were transferred to a side arm of double-side arm Warburg flasks (the other side arm contained ATP), which were immediately gassed with a 95% N_2, 5% CO_2 mixture. The total aerobic exposure of the solutions, which were initially anaerobic, was not more than 3 minutes. Little if any increase in oxidation of epinephrine resulted after the evaluation in the colorimeter. The flasks were anaerobically gassed for 20 minutes. First, epinephrine solutions were tipped; ATP was tipped in 12 minutes later, and the ATPase activity was estimated as before. A 4.4×10^{-6} *M* concentration of autoxidation products estimated as adrenochrome caused a 27% inhibition of ATPase activity, 1.5×10^{-6} *M* caused a 15% inhibition, and detectable effects were observed in concentrations between 10^{-6} *M* to 10^{-7} *M*. These lowest concentrations were not estimated with exactness. The original epinephrine concentration was 6×10^{-5} *M* for most of these assays. However, for one group of assays, 6×10^{-6} *M* epinephrine was used. No inhibi-

tion of ATPase activity appeared attributable to epinephrine itself.

Discussion. Dickens and Glock(10) found 20% inhibition of ATPase activity of myosin from rat skeletal muscle with concentrations of adrenochrome of 10^{-4} *M*. We observed a similar inhibition with concentrations of epinephrine oxidation products of 1.3×10^{-5} *M* and 3×10^{-6} *M*. Detectable effects were observed even at concentrations below 10^{-6} *M*. Adrenochrome, as in the case of other quinones, has been found to oxidize reversibly the –SH groups of several enzymes(11). Dickens and Glock(10) concluded that adrenochrome oxidatively inhibited a rat skeletal muscle myosin preparation and Bailey and Perry(12) have shown that oxidative removal of the –SH groups of myosin inhibited both ATPase activity and ability to combine with actin to form actomyosin. The inhibition observed in these present studies is perhaps related to an action upon –SH groups. The question may be raised whether the prolonged inhibition of uterine motility which is observed following epinephrine treatment *in vivo* and *in vitro*(1) might be related to the enzymatic inhibition observed in these present studies. The possibility of epinephrine oxidation *in vivo* is supported by the work of Wajzer(13), which suggested the existence of an epinephrine-adrenochrome system in mammalian skeletal muscle. Also, Green and Richter(14) have shown that the oxidation of epinephrine *in vitro* is catalyzed by a cyanide-insensitive enzymatic system present in heart and skeletal muscle and by the cytochrome-indophenol oxidase system of heart muscle. However, Schayer and Smiley(15), using isotope technics, concluded that adrenochrome is probably not an intermediate in the metabolism of epinephrine. Nevertheless, their results did not rule out the possibility that in its metabolism, epinephrine might become attached to an enzyme where it is oxidized to adrenochrome, and is then metabolized still further without being released(15). Although it does not seem possible to reconcile the question at this point, the present study suggests that oxidation products of epinephrine, if present, might be expected to have a significant physiological role.

Summary. Oxidation products of epinephrine, estimated as adrenochrome, inhibited ATPase activity of a uterine muscle preparation. The effects were observed with amounts approaching physiological concentrations (10^{-6} *M* to 10^{-7} *M*). No inhibition attributable to epinephrine alone was observed in the range of epinephrine concentrations studied (6×10^{-5} to 6×10^{-6} *M*).

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Effect of Phosphorylated Hesperidin and Hyaluronidase on Absorption From Peritoneal Cavity of Rats.* (23818)

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During experiments unrelated to the study here presented, we observed that an intraperitoneally administered solution of phosphorylated hesperidin, a hyaluronidase inhibitor (1), was not absorbed from the peritoneal cavity for a prolonged period of time. This observation prompted an investigation of the effect of phosphorylated hesperidin and hyaluronidase on absorption of fluid from the peritoneal cavity of rats.

Materials and methods. Hooded female rats (185 ± 15 g) raised at this Institute were employed throughout this study. For determination of fluid absorption from the peritoneal cavity, the following procedure was employed. Rats, under light ether anesthesia, were injected intraperitoneally (I.P.) with 15 ml of a 0.9% NaCl solution employing a 27-gauge hypodermic needle ⅔ inches long. Subsequently, groups of animals were sacrificed at various time intervals following the injection. The peritoneal cavity was exposed surgically, the free fluid recovered by suction and drainage, and then measured. The hyaluronidase† and phosphorylated hesperidin‡ were prepared before each experiment in a 0.9% NaCl solution. All injections were made intraperitoneally. Adrenalectomy, employing the lumbar approach, was performed under ether anesthesia 48 hours before an experiment. The drinking water was replaced after operation by a solution of 0.9% NaCl. All other animals received tap water *ad lib.* during the experiment. Statistical analysis of the data was performed employing the Student's t test.

Results. Reliability of fluid recovery method. To evaluate reliability of fluid recovery technic 16 animals were injected I.P. with 15 ml of 0.9% NaCl solution (normal saline). Animals were sacrificed from 2 to 4 minutes after injection and the fluid recovered and measured. From 93 to 96% of total injected volume could be recovered.

Hesperidin series. Animals were divided into 2 groups. One group received saline solution only, the second received hesperidin and saline, as indicated in Table I (Groups 1 and 2, respectively). Groups of animals were sacrificed 2 minutes to 24 hours following last injection. The results of this experiment are summarized in Fig. 1. For the first 2 hours after 15 ml injection, hesperidin treated animals absorbed the fluid at same rate as untreated animals, but after the second hour absorption rate decreased markedly.

Hyaluronidase series. The effect of hyaluronidase was determined in a similar manner (Table I, Groups 3 and 4, respectively). The animals were sacrificed 2, 3, 6 and 8 hours after 15 ml injection. Data from this experiment, (Fig. 2), show that volume of fluid recoverable from animals treated with hyaluronidase was significantly less than that for saline treated controls.

Hesperidin-hyaluronidase series. It was of interest to determine whether hyaluronidase would counteract the effect of hesperidin. A small but effective dose of hesperidin was considered most practical for this study. A dose response curve was experimentally constructed with this in mind (Fig. 3). From this curve a dose of 5 mg per injection was selected. Animals were divided into 4 groups for this experiment, one group received saline only, the second received hesperidin and saline and the third received hyaluronidase, hesperidin and saline, these two substances being administered with saline in separate injec-

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting views of Navy Department or naval service at large.

† Hyaluronidase was purchased from Worthington Laboratories. The enzyme was of testicular origin supplied in powder form with activity of 175 U.S.P. units/mg.

‡ Phosphorylated hesperidin was donated generously by Dr. G. J. Martin of National Drug Co.

TABLE I. Treatment Schedule for Hesperidin and Hyaluronidase Experiments.

Group	Treatment	No. of animals	Treatment schedule and dosages: Day 1, 9 a.m.	Day 1, 4 p.m.	Day 2, 9 a.m.
1	Saline	95		.25 cc saline	15 cc saline
2	Hesperidin and saline	99		15 mg hesperidin in .25 cc saline	15 mg hesperidin in 15 cc saline
3	Saline	29	.25 cc saline	.25 cc saline	15 cc saline
4	Hyaluronidase saline	29	3000 U.S.P. units hyaluronidase in .25 cc saline	3000 U.S.P. units hyaluronidase in .25 cc saline	3000 U.S.P. units hyaluronidase in 15 cc saline

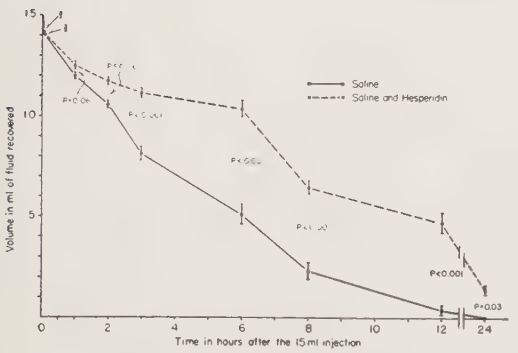

FIG. 1. Effect of hesperidin on fluid absorption from peritoneal cavity of rats.

tions. The fourth group was set up in the same manner as the third, except that a higher dose of hesperidin was employed. Table II shows details of the experimental design and results indicating that hyaluronidase abolished completely the effect of low and almost completely that of high doses of hesperidin.

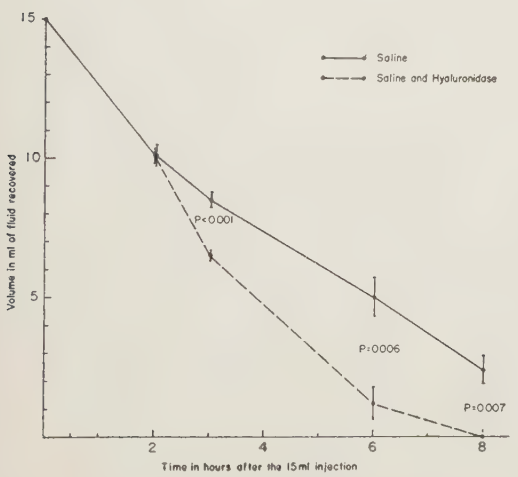

FIG. 2. Effect of hyaluronidase on fluid absorption from peritoneal cavity of rats.

Adrenalectomy series. Four groups of animals were used to test the possibility that hesperidin exerts its effect, *via* the adrenal-pituitary axis, directly or indirectly. One group was adrenalectomized, the second was sham-adrenalectomized and the third and fourth groups were left intact. The first 3 groups were treated with hesperidin in saline, the fourth group received saline only. All animals were sacrificed 6 hours after the 15 ml injection. The schedule of injection dosages and results are summarized in Table III which shows that the effect of hesperidin was not modified by adrenalectomy nor by sham-adrenalectomy.

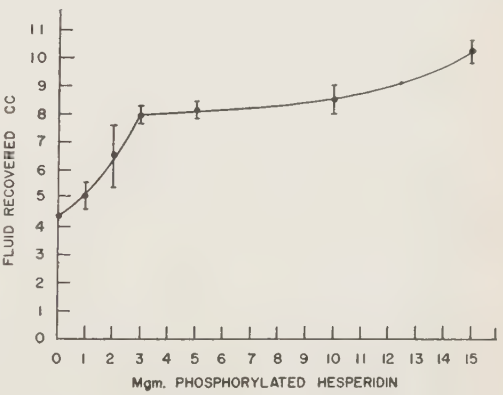

FIG. 3. Effect of various doses of phosphorylated hesperidin on the volume of fluid recovered from the peritoneal cavity of rats 6 hr after an inj. of 15 cc of 0.90% NaCl in distilled water.

Discussion. The mechanism of fluid absorption from the peritoneal cavity presents a multifaceted problem. This problem is magnified by the consideration that the net transfer of an injected solution through the peritoneum and into the circulation probably represents a sum of 2 processes, namely, an in-

TABLE II. Modification by Hyaluronidase of Effect of Hesperidin on Fluid Absorption from the Peritoneal Cavity of Rats.

	Treatment schedule and dosages			Vol of fluid recovered, ml		
	Day 1		Day 2			
Group	9 a.m.	4 p.m.	9 a.m.	4 hr‡	6 hr‡	8 hr‡
1	.25 ml Sal.	.5 ml Sal.	15 ml Sal.	(12)*7.7 ± .35†	(12) 5.2 ± .4	(6) 3.6 ± .64
2	*Idem*	5 mg P.H. in .5 ml Sal.	5 mg P.H. in 15 ml Sal.	(11) 9.6 ± .25 P = .02 Group 1 *vs* 2	(18) 8.8 ± .3 P <.001 Group 1 *vs* 2	(6) 5.5 ± .56 P <.05 Group 1 *vs* 2
3	3000 U.S.P. units HY in .25 ml Sal.	5 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in .25 ml Sal.	5 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in 15 ml Sal.	(6) 6.4 ± .31 P <.001 Group 2 *vs* 3	(12) 2.3 ± .4 P <.001 Group 2 *vs* 3	(6) .4 ± .02 P <.001 Group 2 *vs* 3
4	*Idem*	15 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in .25 ml Sal.	15 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in 15 ml Sal.		(5) 6.5 ± .52 P <.001 Group 2 *vs* 4	

* No. of animals. † Mean ± stand. error of mean. ‡ Hr after 15 ml inj.
P.H. = Phosphorylated hesperidin. HY = Hyaluronidase. Sal. = .9% NaCl sol.

flux of water and solutes into the tissue spaces and blood stream and a return flux in the reverse direction.

Reports in literature as well as findings related in this paper are consistent with the idea that permeability changes of the peritoneum *per se* probably play an important role in the process of absorption. Hyaluronidase produces a marked increase of the permeability of calf peritoneum *in vitro* to normal saline solution(2). Amante and Mittino(3) showed that hyaluronidase increased the absorption of trypan blue from the peritoneal cavity of guinea pigs.

In the present study we have demonstrated, using the net absorption rate as our criterion of fluid absorption, that fluid is absorbed more rapidly in hyaluronidase treated animals. On the other hand treatment with phosphorylated hesperidin markedly retarded the net absorption. That the hesperidin effect is not dependent on presence of adrenals has been shown above (Table III).

The effects of hesperidin were completely abolished by a concomitant administration of hyaluronidase and when an excess of hyaluronidase was employed the absorption was faster than in the saline controls.

Our *in vivo* observations are paralleled by *in vitro* study of the effect of hyaluronidase and phosphorylated hesperidin on the permeability of a mouse connective tissue membrane(4), showing that hyaluronidase increases the permeability and hesperidin reduces it to the level found before exposure to hyaluronidase. Furthermore it was demon-

TABLE III. Effect of Hesperidin on Fluid Absorption from the Peritoneal Cavity of Adrenalectomized Rats.

Group	No. animals	Treatment schedule and dosages			Fluid recovered*(ml)	P-values
		Day 1—9 a.m.	Day 2—4 p.m.	Day 3—9 a.m.		
1	10	Adrenalectomy	5 mg P.H. in .25 ml Sal.	5 mg P.H. in 15 ml Sal.	8.3 ± .4†	P = .82 Group 1 *vs* 3
2	5	Sham-adrenalectomy	*Idem*	*Idem*	7.9 ± .5	P = .55 Group 2 *vs* 3
3	10		"	"	8.2 ± .3	P <.001 Group 3 *vs* 4
4	12		.25 ml Sal.	15 ml Sal.	4.4 ± .4	

* Six hr after 15 ml inj. † Mean ± stand. error of mean.
P.H. = Phosphorylated hesperidin. Sal. = .9% NaCl sol.

strated in these studies that hesperidin alone reduces permeability of the connective tissue membrane. In view of the quoted evidence the possibility that hesperidin alters the absorption process by a direct effect on the membranes rather than by inhibition of hyaluronidase deserves serious consideration. Also, sight should not be lost of the possibility that hesperidin decreases the net absorption not by blocking nor diminishing the transfer of the fluid out of the peritoneal cavity, but by increasing the return flux from the circulatory system and tissue spaces into the peritoneal cavity.

Summary. 1. The effect of hyaluronidase and phosphorylated hesperidin on absorption of 0.9% NaCl solution from the peritoneal cavity of female rats was studied. 2. Intraperitoneal administration of hyaluronidase increased and administration of phosphorylated hesperidin decreased the net fluid absorption. 3. Hyaluronidase abolished the effect of hesperidin when administered to hesperidin treated animals. 4. Adrenalectomy had no effect on the action of hesperidin. 5. The possibility that the hesperidin decreases the net absorption in untreated animals by direct effect on permeability of the membranes rather than by inhibition of hyaluronidase is discussed.

The authors wish to express their appreciation to Dr. E. P. Vollmer and Dr. J. J. Christian for valuable advice, and to Captain H. Sudduth, MC, USN for his splendid cooperation and interest in this work.

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Utilization of Vit. A and Carotene by Normal and Deficient Sheep.* (23819)

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For many years, the utilization of Vit. A and carotene has been extensively investigated in monogastric animals but to a limited degree in ruminants. It is well known that there is a marked specie difference regarding the relative values of carotene and types of Vit. A. Results recently reported(9) have indicated a depressed carotene utilization by the Vit. A deficient bovine. Furthermore, unpublished results from our laboratory have shown that the value of carotene for the bovine, as measured by hepatic Vit. A storage, was greatly influenced by the initial Vit. A content of the liver. Therefore, it was of interest to elucidate the suggested impairment of carotene utilization by the deficient animal as well as to establish relative biological values of types of Vit. A and carotene for sheep.

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Method. Nineteen crossbred yearling wethers were divided into 2 groups of 9 and 10 animals each and pretreated with diets adequate and deficient in carotene respectively for 232 days. Four days prior to and during the experimental period all animals were fed a carotene deficient ration of barley straw and cottonseed meal. Aqueous solutions of beta carotene, Vit. A alcohol and Vit. A acetate were prepared, immediately preceding administration by the following method: One gram of the respective materials was dissolved in 20 ml chloroform; 25 ml Tween 80 were added and the chloroform was removed under vacuum at 62°C. Each remaining solution was diluted with distilled water to give a final concentration of 2 mg/ml. Trial A: Within 6 hours following liver biopsy, half of the normal and half of the deficient sheep were injected, intraruminally, with 0.6 mg of beta carotene/lb body weight. The re-

TABLE I. Hepatic Vit. A Storage in Normal and Deficient Sheep following Administration of Beta Carotene and Vit. A Alcohol and Acetate.

	Treatments		
	Beta carotene	Vit. A alcohol	Vit. A acetate
Trial A			
Normal			
No. of wethers	5	4	
Avg wt, lb	94.8	103.5	
*Avg hepatic vit. A:			
Initial content	34.82	41.08	
Final "	53.93	104.26	
Storage	19.11	63.18	
Deficient			
No. of wethers	5	5	
Avg wt, lb	88.1	84.4	
*Avg hepatic vit. A:			
Initial content	3.58	3.97	
Final "	5.57	13.19	
Storage	1.99	9.22	
Trial B			
No. of wethers		5	5
Avg wt, lb		84.6	81.1
*Avg hepatic vit. A:			
Initial content		9.44	9.75
Final "		28.00	17.78
Storage		18.56	8.03

* Expressed as μg vit. A/g fresh liver.

maining animals were administered with the same amount of Vit. A alcohol. Blood, for Vit. A determination, was withdrawn from the jugular vein of each animal just prior to carotene and Vit. A administration. Following treatment, blood samples were collected every 6 hours for 2 days and daily thereafter for 4 days. Liver samples were again obtained from all sheep 6 days following the carotene and Vit. A administration. Hepatic Vit. A storage, as influenced by treatment, was determined from differential liver analyses. Trial B: The 10 deficient wethers used in Trial A, in which half received beta carotene and half Vit. A alcohol, were allotted on the basis of hepatic Vit. A content into groups of 5 animals each. Vit. A alcohol was injected intraruminally at the rate of 0.6 mg/lb body weight to one group while, similarly, Vit. A acetate was administered to the second group. These injections were performed within 6 hours after the second biopsy of trial A. Blood was withdrawn for Vit. A determinations at 0, 12, 18, 24, 36, and 48 hours following treatment. Hepatic Vit. A storage, resulting from the treatments, was determined from liver samples obtained 4.5 days following Vit. A administration. Plasma and liver Vit. A determinations were performed as described by Kimble(1) and Gallup and Hoefer(2) respectively.

Observations. Based on numerous studies with rats, the Expert Committee on Biological Standardization(3) reported that on an equal weight basis, beta carotene was equivalent to half the amount of Vit. A alcohol. However, Table I shows that normal wethers treated with Vit. A alcohol stored 3.3 times more hepatic Vit. A than similar animals treated with beta carotene.

Fig. 1 shows that Vit. A absorption commenced within 6 hours following Vit. A alcohol administration and attained maximum absorption at 12 hours. The Vit. A was not converted or absorbed from beta carotene until 18 hours, followed by a slower more sustained plasma Vit. A level as shown in Fig. 1. The difference in the rapidity of absorption may be accounted for by the possible difference in the intestinal site of Vit. A alcohol absorption and beta carotene conversion. Absorption of Vit. A alcohol has been shown to take place throughout the entire small intestine(4) whereas carotene possessed a maximum conversion in the middle of the intestine and no conversion anterior to the entrance of the bile duct(5).

Moore's(6) study indicated that rats were capable of storing only 200 i.u. of Vit. A daily from massive doses of carotene, while later work(7) showed that rats readily stored 20,-

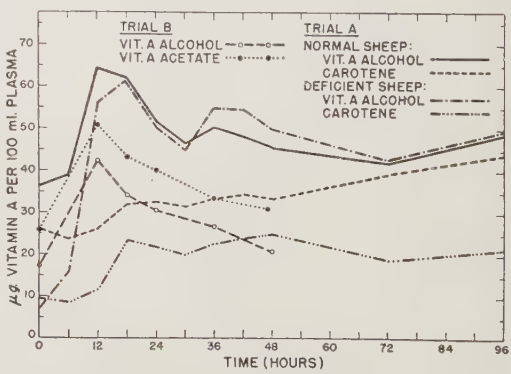

FIG. 1. Absorption rates of Vit. A as influenced by administration of beta carotene and Vit. A alcohol and acetate.

000 i.u. from a dietary Vit. A source. Therefore, wethers may perform similar to rats in that only a certain amount of carotene may be converted regardless of the magnitude of intake.

It has been shown by Lemley *et al.*(8) with rats that the efficiency of hepatic storage of Vit. A decreased with increasing levels of injected Vit. A. Therefore, a greater hepatic storage differential between beta carotene and Vit. A alcohol treatment may have existed if the efficiency of hepatic deposition had been in accord with the two respective levels and rates of absorption (Fig. 1).

The liver storage of Vit. A, that resulted from beta carotene and Vit. A alcohol administration in deficient wethers was markedly lower than in similarly treated normal animals. The increase of plasma Vit. A in the deficient wethers (Fig. 1), may account for the difference in the liver storage between the normal and deficient sheep treated with Vit. A alcohol. While the hepatic Vit. A storage was 6.8 times greater in normal than in deficient sheep treated with Vit. A alcohol, normal wethers, administered beta carotene, stored 9.1 times more hepatic Vit. A than carotene-treated deficient animals.

The decreased utilization of beta carotene by the deficient wethers was not only indicated by the low hepatic Vit. A storage but also by the low plasma Vit. A levels (Fig. 1). At no time during this study did the plasma Vit. A in the carotene deficient sheep equal the amount in normal animals. Since Vit. A alcohol absorption by deficient wethers was not depressed, these results indicate that Vit. A deficiency may possibly impair the rate of carotene conversion. Furthermore, the ratio of biological value of Vit. A alcohol to beta carotene was 3.3 in the normal animals and 4.6 in the deficient. These results are similar to those reported by Erwin *et al.*(9) where depressed carotene utilization was noted in Vit. A deficient steers. While Week and Sevigne (10) reported higher absorption rates for the alcohol compared to the acetate form of Vit. A in humans, the same workers(11) found that Vit. A acetate was superior to Vit. A alcohol in rats.

Although Fig. 1 shows a very similar absorption rate for alcohol and acetate forms of Vit. A in wethers, the respective hepatic storage values shown in Table I differed during trial B. Hepatic storage of Vit. A was 2.3 times greater in sheep treated with Vit. A alcohol than those treated with Vit. A acetate.

Summary. Vit. A alcohol attained a maximum absorption at 12 hours post treatment, while beta carotene was absorbed at a slower more sustained rate. On the other hand, Vit. A alcohol and acetate were absorbed at comparable rates. The comparative value of Vit. A alcohol and beta carotene as measured by hepatic storage was markedly different for normal and Vit. A deficient sheep indicating that deficiency may impair carotene utilization.

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Effect of Age upon Collagen and Hexosamine Content of Rat Skin.* (23820)

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Sobel and his co-workers have found that the hexosamine and collagen content of rat skin increased with rat body weight(1). They have also shown that, since the collagen concentration increased more rapidly than the hexosamine concentration, the ratio of hexosamine to collagen decreases with age(2). We have studied these components in the skin of animals weighing more than 250 g, the upper weight limit used by Sobel *et al.* The results of the analyses for the hexosamine, hydroxyproline and nitrogen contents of rat skin are reported below.

Materials and methods. Preparation of tissue. Wistar strain female rats ranging in weight from 50 to 500 g were grouped into 7 weight classifications, with 6 rats in each group. These animals were fed on Fox Chow pellets *ad lib.* After killing by etherization, a section of dorsal skin of each rat was shaved, and about 2 square cm of tissue removed. This tissue was carefully dissected clean of adhering fat and muscle, and stored in frozen state. After thawing, tissues were weighed, minced and autoclaved with 10 ml/g of 4 N HCl for 3 hours. The samples were then incubated at 100°C for 2 hours, at which time duplicate 0.1 ml samples were removed for nitrogen and hydroxyproline determination. After 5 hours incubation, 1 ml samples were removed for hexosamine analysis. These heating times were optimal for hydrolysis to hexosamine and hydroxyproline, while quantitative recoveries of these compounds could be affected from the tissue mixture. The results of these analyses were expressed in μmoles/g of fresh, wet, tissue. *Analysis for hexosamine.* The 1 ml samples were neutralized with 4 N NaOH and diluted with 2 ml of acetyl acetone-carbonate reagent(3) and 1 ml of 4 N NaCl. This mixture was treated according to Blix(3). The results were translated *via* a standard curve into μmoles/g fresh tissue. *Analysis for nitrogen.* The appropriate 0.1 sample was diluted 300 fold with water and 0.1 ml of this solution was mixed with 0.2 ml of a digestion mixture of 50% sulfuric acid containing 1% SeO_2. After digestion this mixture was diluted to 7 ml with water and 3 ml of Koch and McMeekin Nessler solution was added. The resulting optical density at 430 mμ was translated *via* a standard curve into μmoles/g of fresh tissue. *Analysis for hydroxyproline.* 0.1 ml samples were diluted to 2.5 ml with a 4 N HCl. 0.5 ml of this solution was diluted to 5 ml with water and 1 ml samples were then analysed for hydroxyproline by the method of Martin and Axelrod(4).

Results. The mean weight of each group of rats was 65, 200, 240, 280, 330 and 450 g. The correlation between body weight, and hence the age of rat skin, and mean concentration of hydroxyproline, hexosamine and nitrogen/g of tissue and standard deviation of each group is shown in Fig. 1. Although the amount of hydroxyproline increased with age in animals weighing up to 280 g, there was a statistically significant decrease in concentration of this component with animals of greater weight. There was essentially no change in amount of hexosamine/g of fresh rat skin over the whole range of animal weights studied. The change in total nitrogen of whole tissue closely paralleled the hydroxyproline determination. The ratio of hexosamine to hydroxyproline concentration declined in animals weighing from 65 to 330 g. Above this weight however, the ratio increased significantly.

Since variation in water content of connective tissue occurs with age, it was decided to relate hexosamine and hydroxyproline content of rat skin to total nitrogen of tissue. The results are illustrated in Fig. 2. The ratio of hexosamine to hydroxyproline was naturally unchanged. The ratio of hydroxyproline to nitrogen was changed only in that the maximum occurred in animals weighing 240 g. The shape of curve illustrated in Fig. 2 was

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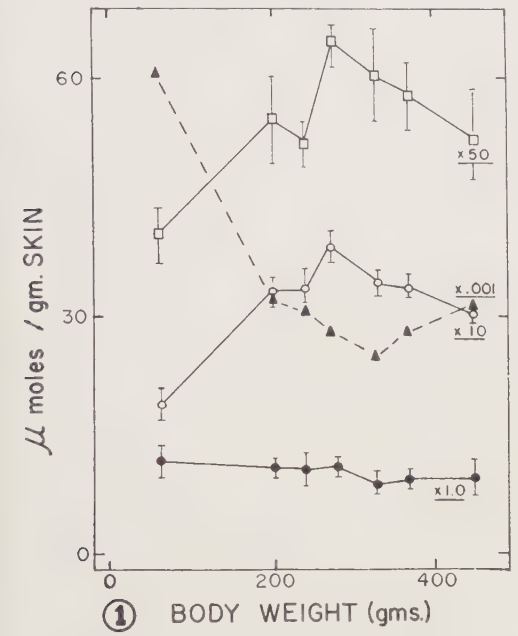
relatively unchanged from that presented in Fig. 1. The effect of animal weight upon hexosamine to nitrogen ratio as shown in Fig. 2, was profoundly altered from that described in Fig. 1. Concentration of hexosamine relative to that of nitrogen decreased with body weight up to 330 g. Above this weight size, however, the ratio increased with further increases in animal body weight. These changes were closely paralleled by the effect of body weight upon hexosamine to hydroxyproline ratio.



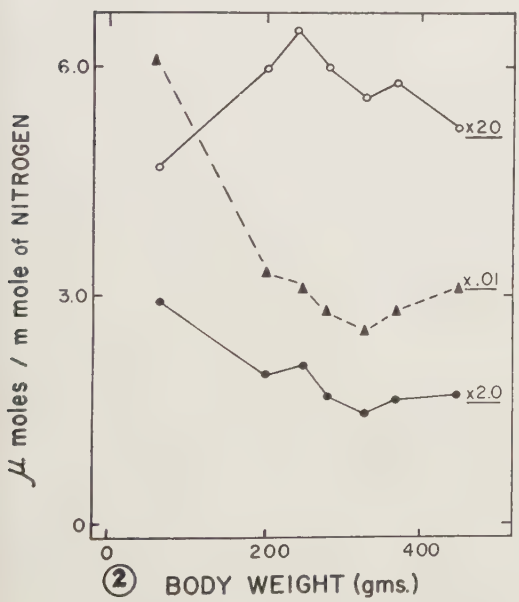


FIG. 1 (top). Effect of body wt upon concentration of hexosamine (●), hydroxyproline (○) and nitrogen (□) in rat skin, and upon ratio of hexosamine to hydroxyproline (▲). Values expressed per g of fresh whole skin.

FIG. 2 (bottom). Effect of body wt upon ratio of hexosamine (●) and of hydroxyproline (○) to total tissue nitrogen, and upon ratio of hexosamine to hydroxyproline values expressed per μmole of nitrogen (▲).

Discussion. Contrary to data presented by Sobel and his co-workers(1,2) in their study of rats weighing to 250 g, we have been unable to find any significant change in hexosamine content of skin, unless the results are expressed relative to total nitrogen of tissues. If the data are so presented then the concentration of hexosamine does not increase but rather decreases in the skin of rats up to about one year in age. With animals older than this (body weight greater than 330 g) the relative concentration of hexosamine increases. The close correlation between effect of age upon relative hexosamine content of rat skin and the ratio of hexosamine to hydroxyproline indicates that formation of mucopolysaccharides may be a critical feature of the ageing process. The parallel response of hydroxyproline and tissue nitrogen to age suggests that there is no disproportionate alteration in skin proteins with increasing body weight.

The presentation of hexosamine and hydroxyproline data relative to the total nitrogen of tissue has some obvious advantages; a) the tedious fat and water extraction procedures are not required, b) concentrations of the components are expressed relative to total protein of tissue, and c) a third component, extraneous protein, is thereby indirectly measured.

Summary and conclusions. 1) Concentration of hexosamine in whole rat skin is relatively unaffected by animal age, although the amount of this material relative to total tissue protein is progressively decreased with increasing body weight up to 330 g. Both the total and relative concentrations of hydroxyproline in rat skin are increased with animal weight up to 280 or 240 g respectively. Above this weight class the amount of hydroxypro-

line decreased slightly. Total tissue nitrogen parallels these changes. 2) Above 330 g however, the relative hexosamine concentration increases with body weight. The effect of body weight upon the ratio of hexosamine to hydroxyproline closely parallels the response of relative concentration of hexosamine to rat age.

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Seizure Patterns in Young Animals. Significance of Brain Carbonic Anhydrase. II.*† (23821)

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Previous work has demonstrated a direct relation between the activity of brain carbonic anhydrase and the susceptibility of animals to experimental seizures. In mice, acetazolamide has been shown to be approximately twice as potent as sulfanilamide as an inhibitor of brain carbonic anhydrase and as an anticonvulsant; the maximum degree of inhibition of brain enzyme corresponded with the time of peak anticonvulsant effect of each drug(1). In addition, it has been shown recently in newborn animals of different species that the development of susceptibility to various experimental seizure patterns is directly related to the level of carbonic anhydrase in the brain(2). In rats between 20 and 35 days of age, in which a high level of brain carbonic anhydrase was observed, it was possible to induce both major and minor seizure patterns by variation of the strength of an electroshock stimulus. A current of 50 m. amps. applied for 0.2 sec. was the threshold stimulus required for induction of the maximal seizure pattern, which consists principally of tonic extension of the hind-limbs (grade 5); tonic flexion or extension of the forelimbs with clonus (grade 4) was induced by a stimulus of 40 m. amps.; and symmetrical clonic seizures with loss of posture (grade 3) occurred in response to a threshold current of 30 m. amps. Stimuli of 20 and 10 m. amps. respectively were required for the induction of catatonic (grade 2) and hyperkinetic (grade 1) minor seizure patterns. In rats younger than 20 days, the grade of seizure pattern obtained in response to an electroshock stimulus of 50 m. amps. was less severe than that observed in older rats between 20 and 35 days of age, and the level of brain carbonic anhydrase was correspondingly lower. In the newborn, a hyperkinetic grade 1 seizure response was associated with a minimal amount of carbonic anhydrase in the brain. With a rapid increase in enzyme activity between 10 and 20 days, seizure patterns of grades 2, 3 and 4 were obtained.

In the present investigation, designed to test further this apparent relation between carbonic anhydrase activity in the brain and various patterns of seizure activity, the anticonvulsant effect of acetazolamide has been observed in parallel with estimations of the degree of inhibition of the enzyme.

Methods. Effect of acetazolamide on seizure patterns. The effect of acetazolamide on seizure patterns induced by electroshock was determined in albino rats (Holtzman strain) between 28 and 32 days of age and weighing between 75 and 100 g. Acetazolamide sodium was dissolved in distilled water and administered by subcutaneous injection. Since tolerance develops on repeated injection(3,4), in-

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† Acetazolamide (Diamox) was supplied by Lederle Medical Research Department, Pearl River, N. Y.

dividual animals were tested only once. The susceptibility of treated animals to grade 5 and grades 3 and 4 seizure patterns was tested by electroshock stimuli of 100 m. amps. and 60 m. amps. respectively, applied for 0.2 sec. by corneal electrodes. These stimuli were approximately twice threshold intensity. A current of 60 m. amps. was used also to test for grades 1 and 2 minor seizure responses in treated animals. With these stimuli, a seizure response of a grade not less than that under investigation would be obtained in 99% of control animals untreated with acetazolamide. In a preliminary experiment, the anticonvulsant activity of acetazolamide in doses between one and 1000 mg/kg was observed in five groups of animals tested at an interval of one and one-half hours after administration of the drug. Since the approximate doses required to protect rats against the various grades of seizure patterns varied widely and since it was necessary to give solutions of different concentration, the time of peak anticonvulsant effect was determined separately at two dose levels of 5 and 400 mg/kg. In each instance, 8 groups of 4 animals were tested at intervals of one half-hour for a period of 4 hours after receiving the drug.

To compare the relative potency of acetazolamide against the various seizure patterns, the effects of graded doses were determined in a total of 65 animals at the times of peak effect, until points were established between complete and no protection. For each seizure pattern of grades 2 to 5, results obtained were plotted on logarithmic probability paper, and a regression line fitted to the plotted points by eye. From these plotted data, values for ED_{50} were determined and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(5). The effect of acetazolamide on seizure patterns of rats aged 12 to 14 days and weighing between 15 and 20 g was studied also. Five groups of 4 animals were given acetazolamide sodium by intraperitoneal injection in doses of 10, 50, 100, 200, and 500 mg/kg in a volume of 0.2 to 0.5 ml. A control group received an equal volume of 0.85% sodium chloride solution. All animals were tested with an electroshock stimulus of 50 m. amps. for 0.2 sec. at an interval of one and one-half hours after receiving the drug.

Estimation of degree of inhibition of brain carbonic anhydrase. Rats of the same Holtzman strain, aged 29 days and weighing between 70 and 85 g, were given acetazolamide sodium by subcutaneous injection in doses of 2.5, 10, 100, and 1000 mg/kg. At times of peak anticonvulsant effect, as determined previously for both small and large dose ranges, the rats were killed by decapitation. Larger blood vessels and macroscopic blood were removed from the cerebral cortex and white matter, and the brain tissue was weighed and then homogenized with 0.85% sodium chloride solution in a dilution of 1:10. In previous experiments, enzyme activity in contaminating red cells had been estimated and found to be less than one per cent of that in the specimen of brain tissue prepared in this manner. After centrifugation, the carbonic anhydrase activity of the supernatant saline extract was estimated by the manometric method of Meldrum and Roughton(6). All observations were made at atmospheric pressure and at 0°C. The rate of evolution of carbon dioxide was corrected for the limiting effect of diffusion through the gas-liquid interface(7) and the concentration of active enzyme was calculated from the corrected rate. Three animals were tested at each dose level of acetazolamide, and the amount of free enzyme [E] in the brain extracts prepared from these animals was compared with the total enzyme activity [ΣE] in brain extracts prepared from 6 control untreated animals. The concentrations of inhibitor in the reaction vessel and in the undiluted brain tissue and the degree of enzyme inhibition at body temperature were then calculated, the dissociation constants for acetazolamide at 0°C and at 38°C being employed(1,8). The concentration of inhibitor was referred to brain water which, in rats aged 28-32 days, is 80% of the weight of the whole brain(2). The inhibition of carbonic anhydrase by some sulfonamide inhibitors has been studied by Davenport(9). The mass action expression for the reaction between carbonic anhydrase and its inhibitors is $\frac{[E]\,([\Sigma I]-[EI])}{[EI]} = K$ where [E] is the enzyme concentration in presence of inhibi-

tor, $[\Sigma I]$ the total concentration of inhibitor in the reaction mixture, $[EI]$ the concentration of enzyme-inhibitor complex, and K the dissociation constant. In calculations of the concentration of inhibitor in the brain sample, the assumption that the mass action equation is valid in the body tissues is implicit. $[E]$ and $[EI]$ were measured in terms of enzyme activity; since purified carbonic anhydrase was unobtainable, the absolute value of the enzyme unit could not be defined, and it was necessary to regard the expression $([\Sigma I] - [EI])$ as equal to $[\Sigma I]$. However, with acetazolamide, a strong inhibitor, the amount bound to the enzyme may not be negligible with respect to the total inhibitor concentration and, by this indirect method of calculation, a degree of error may be unavoidable. For this reason, the concentrations of inhibitor in the brain extracts were estimated directly by a biological assay for comparison with the values calculated indirectly. The brain extracts were heated to 100°C in a boiling water bath for 5 min. to destroy the enzyme, and a known volume of the extract which contained inhibitor was added to a known amount of brain carbonic anhydrase in the reaction vessel. From the percentage inhibition of enzyme observed at 0°C, the concentration of acetazolamide in the vessel was read from the data in Fig. 1, in which percentage enzyme inhibition is plotted against total inhibitor concentration. The concentration of inhibitor in the brain was calculated and, by use of the appropriate constant for acetazolamide, the percentage inhibition of brain carbonic anhydrase at body temperature was then estimated. Thus, 2 independent measurements of inhibitor concentration in the brain were utilized in the determination of percentage inhibition of the enzyme at 4 different dose levels of acetazolamide.

Results. Anticonvulsant effects of acetazolamide. In rats aged 28 to 32 days, the time of peak anticonvulsant effect of acetazolamide sodium varied with the dose. At 5 and 400 mg/kg, the times of peak effect were one hour and 2 hours respectively. The values for ED_{50} of acetazolamide against the various grades of electroshock seizure patterns are shown in Table I, with 95% confidence limits in parentheses. The seizure response of each individual animal treated with acetazolamide is recorded in Fig. 2.

While the hind-limb tonic extensor component of the grade 5 seizure was abolished by very small doses of acetazolamide, large doses were required to protect animals from tonic flexion or extension of the fore-limbs (grade 4 seizures), from generalized severe clonus and loss of posture (grade 3), and

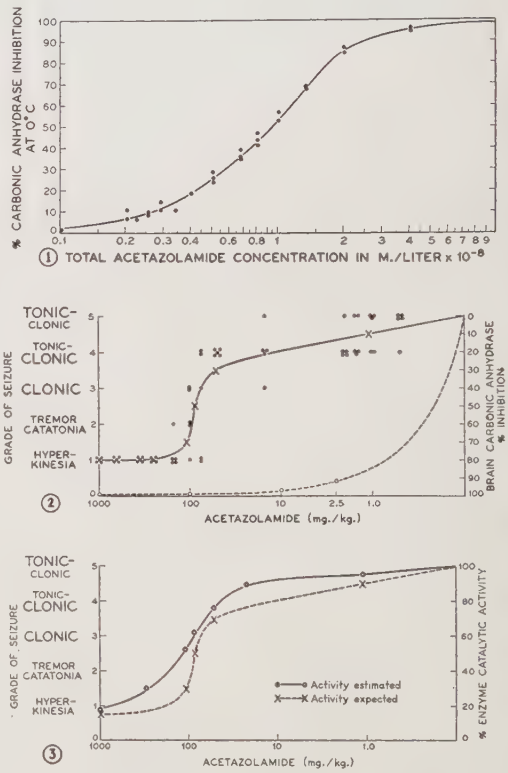

FIG. 1. Inhibition of carbonic anhydrase by acetazolamide at 0°C.

FIG. 2. Seizure patterns of rats aged 29 days and percentage inhibition of brain carbonic anhydrase in relation to dose of acetazolamide sodium. Points represent the seizure responses of individual test animals, and crosses, the values of the ED_{50} of acetazolamide for various seizure patterns.

FIG. 3. Susceptibility to seizure patterns in relation to functional activity of carbonic anhydrase in the brain of young rats. ○——○, mean values calculated from percent fractional enzyme activity observed in presence of acetazolamide and from the data of Davenport, H. W.(9). ×---×, mean values determined from enzyme activity observed in relation to development of susceptibility to seizure patterns.

TABLE I. Percentage Inhibition of Brain Carbonic Anhydrase and of the Reaction Catalyzed by the Enzyme in Rats Protected from Seizure Patterns by Acetazolamide.

100% susceptibility to seizures: Electroshock seizure pattern		Brain carbonic anhydrase		50% protection from seizures: Inhibition of enzyme expected,* %	ED_{50} acetazolamide in rats with 125 units enzyme, mg/kg	Inhibition of enzyme estimated, %	Inhibition of catalyzed reaction calculated,† %
Grade	Type	Units	%				
5	TONIC-Clonic	125	100	**10**	1.1(0.7– 1.8)	86.0 (80 –90)	**5** (4– 6)
4	Tonic-CLONIC	100	80	**30**	51 (32 – 81)	99.0 (98.6–99.4)	**24** (16–36)
3	CLONIC	75	60	**50**	86 (61 –120)	99.4 (99.2–99.6)	**38** (28–52)
2	Tremor-Catatonia	50	40	**70**	107 (79 –145)	99.6 (99.4–99.7)	**48** (36–58)
1	Hyperkinesia‡	20	16	**84**		99.98	83

* The reciprocal of the level of enzyme activity observed in relation to development of susceptibility to seizure patterns. (Confidence limits not calculated owing to small No. of samples at individual levels of seizure activity.)

† Calculated from percent enzyme inhibition observed in presence of acetazolamide and from the data of Davenport, H. W.(9). Mean values, with 95% confidence limits in parentheses.

‡ Not modified by acetazolamide, 1000 mg/kg, and 99.98% inhibition of enzyme.

from the tremor and catatonia of grade 2 seizures. Grade 1 electroshock seizure patterns, which consist of violent hopping and running movements but without loss of posture, were refractory to acetazolamide, even in doses up to 1 g/kg.

In rats aged 14 days, the electroshock seizure patterns of acetazolamide-treated animals were not significantly different from those of control animals. The animals in all groups responded with a grade 1 hyperkinetic seizure pattern.

Relation of seizure pattern to brain carbonic anhydrase. The percentage inhibition of brain carbonic anhydrase at body temperature, shown in Table II, was calculated from the concentration of acetazolamide in the brain, estimated by the two methods described.

By direct assay, the degree of enzyme inhibition at 0°C was lower than that observed *in vitro* by the indirect determination. As possible explanations for this discrepancy of results, either the acetazolamide had been destroyed partially by boiling or, in the absence of enzyme, the inhibitor had become adsorbed on the deposit of the centrifuged brain extract. The higher values calculated by the indirect method were therefore accepted as more nearly correct than those obtained by direct bioassay.

In Fig. 2, the degree of inhibition of brain carbonic anhydrase and the modified seizure responses of 29-day-old rats are plotted in re-

TABLE II. Inhibition of Brain Carbonic Anhydrase in Relation to Dose and Brain Concentration of Acetazolamide.

	Indirect assay				Direct assay			
Dose of acetazolamide, mg/kg	Enzyme inhibition *(in vitro)* % at 0°C	Conc. of acetazolamide, M/l × 10^{-7}	Enzyme inhibition *(in vivo)* % at 38°C		Enzyme inhibition *(in vitro)* % at 0°C	Conc. of acetazolamide, M/l × 10^{-7}	Enzyme inhibition *(in vivo)* % at 38°C	
2.5	33	13	92.4		12	8	88.5	
	32	12	92.0	92.4	6	6	84.1	87.0
	34	13	92.8		12	8	88.5	
10	49	25	95.8		27	14	93.2	
	70	60	98.3	97.1	19	11	91.6	92.3
	59	38	97.2		22	12	92.3	
100	89	204	99.5		82	50	97.9	
	90	239	99.6	99.5	81	45	97.8	97.8
	90	226	99.5		76	45	97.7	
1000	99.3	3444	99.97		99.7	>275	99.6	
	99.5	6690	99.98	99.98	99.5	"	99.6	>99.6
	99.9	25190	99.99		99.2	"	99.6	

lation to acetazolamide at various dose levels. Table I shows the percentage inhibition of carbonic anhydrase in the brain in relation to the grade of seizure pattern observed in animals treated with acetazolamide. When 86% of the brain enzyme was inhibited, grade 5 tonic seizures were prevented in 50% of animals tested; with 99.4% inhibition, grade 3 clonic seizures were abolished in a similar proportion of animals. The grade 1 hyperkinetic seizure pattern was not controlled despite the inhibition of brain enzyme by 99.98%.

Discussion. In a previous study(2), the development of susceptibility to various seizure patterns has been correlated with the concentration of carbonic anhydrase in the brain of rats between 1 and 35 days of age. In this age group, rats of 25 to 35 days have the maximal degree of enzyme activity, equivalent to a mean value of 125 units/g/wet weight of brain; and this 100% enzyme activity is correlated with complete susceptibility to the grade 5 maximal seizure pattern. The lower levels of enzyme activity which correspond with susceptibility to less severe grades of seizure patterns are recorded in Table I. From these data, the percentage inhibition of enzyme activity expected to correspond with 50% protection from each seizure pattern has been calculated. For example, when 50% of animals are protected from the grade 5 tonic seizure, the expected degree of enzyme inhibition would be 10%. However, in response to the ED_{50} of acetazolamide for this seizure pattern, the degree of enzyme inhibition observed was approximately 86%.

While the percentage inhibition of carbonic anhydrase was inversely related to the severity of the modified seizure patterns, the degree of inhibition of the enzyme necessary to afford protection from seizures was much greater than that expected from estimations based on developmental studies of enzyme activity and seizure susceptibility. In addition, in rats treated with acetazolamide in doses up to 1 g/kg, the grade 1 hyperkinetic seizure pattern was completely refractory, despite the inhibition of brain carbonic anhydrase to a level of 0.02% of normal.

Observations by Davenport(9) have shown that the inhibition of the hydration or dehydration of carbon dioxide and of the catalyzed reaction is not proportional to the inhibition of carbonic anhydrase. The rate of uptake of carbon dioxide by blood was studied in the presence of thiophene-2-sulfonamide and sulfanilamide. It was reported that in order to reduce the rate of the catalyzed reaction by 10% at 0°C the carbonic anhydrase was inhibited by more than 98%; and at body temperature, the concentration of inhibitor required to effect this degree of inhibition would be much higher. From a plot of these data and the percentage inhibition of brain carbonic anhydrase observed in response to acetazolamide, the corresponding values for the degree of inhibition of the reaction catalyzed by the enzyme were calculated and recorded in Table I. The reciprocals of these values, or the levels of functional activity of the enzyme which are correlated with susceptibility to the various grades of seizure patterns, are shown in Fig. 3.

That the functional activity of the enzyme observed in animals treated with acetazolamide is of a similar order of magnitude to that expected from estimations based on developmental studies of enzyme activity is suggested by the comparable distributions of the mean values obtained. A more exact correlation may have been observed had the estimations of the rate of the catalyzed reaction been possible in rats of all age groups between 1 and 35 days.

Thus, it is possible to reconcile the apparent discrepancy between the degree of enzyme inhibition observed in relation to suppression of various seizure patterns by acetazolamide and that expected from the known levels of brain enzyme which are correlated with development of seizure susceptibility. In addition, the failure of acetazolamide and a high degree of enzyme inhibition to control the grade 1 hyperkinetic seizure pattern may be explained by the persistence of the reaction catalyzed by brain carbonic anhydrase at a rate sufficient for the propagation of a minor seizure discharge. The functional significance of brain carbonic anhydrase in the mechanism of the seizure process and in the development of susceptibility to seizure patterns in young animals is strongly suggested by the data pre-

sented.

Summary. The relation between activity of brain carbonic anhydrase and susceptibility to various grades of seizure patterns has been studied in young rats by observations of the effects of acetazolamide. The percentage inhibition of carbonic anhydrase and of the reaction catalyzed by the enzyme is related to the suppression of tonic and clonic seizure patterns. It is suggested that brain carbonic anhydrase and its specific catalytic activity may be essential for the propagation of the seizure discharge. Low levels of enzyme activity may be sufficient for focal discharge and the induction of minor seizure patterns while a relatively high degree of activity may be required for the propagation of a generalized seizure discharge and the induction of a major tonic seizure.

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Tetramethylrhodamine as Immunohistochemical Fluorescent Label in the Study of Chronic Thyroiditis.* (23822)

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Since development by Coons and his coworkers(1) of the fluorescent immunohistochemical technic using fluorescein groups as the fluorescent label, there has been interest in developing fluorescent labels of a color other than the fluorescein apple green. Thus, Clayton(2) reported labelling antibodies with 1-dimethylaminonaphthalene-5-sulfanyl chloride and nuclear fast red to produce yellow and red fluorescence, respectively. Silverstein(3) recently reported that tetraethylrhodamine can be coupled through ureido groups to protein to yield a label which fluoresces orange in ultraviolet light. We had been working on the fluorescent label problem also and had prepared the tetramethylrhodamine isocyanate which when coupled to protein fluoresces orange. We are reporting the use of this label in a study of the serum components associated specifically with chronic thyroiditis (Hashimoto's disease). Roitt, Doniach, Campbell and Hudson(4) reported that serum of patients with Hashimoto's disease contains material capable of precipitating with human thyroid extract. Witebsky, Rose, Terplan, Paine and Egan(5) have shown that serum of patients with chronic thyroiditis agglutinates tanned sheep red cells coated with human thyroid extract. Previously, Witebsky and Rose(6,7) had shown that thyroiditis similar to that in humans can be produced in rabbits by injecting animals with thyroid extract, either from same species, or from a portion of individual's own thyroid.† From these observations, it has been postulated that chronic thyroiditis in humans may be the result of an auto-immunization process in which an individual produces antibodies against tissue components in his own thyroid. The se-

* This work was supported by the U. S. Public Health Service through grant of Nat. Cancer Inst. and Nat. Heart Inst.

† Beutner, Witebsky, Rose and Gerbasi(8) have shown that rabbit thyroid is stained by fluorescein labelled sera from rabbits injected with rabbit thyroid.

rum components in a patient with chronic thyroiditis have been shown to be in the rapidly sedimenting globulin fraction(10). To determine which structures of the thyroid gland combine with precipitating chronic thyroiditis serum, we treated sections of normal human thyroid with such serum and determined where combination took place by staining subsequently with tetramethylrhodamine (and fluorescein) labelled rabbit anti-human gamma globulin antiserum. Similarly, we demonstrated that thyroid tissue from a patient with chronic thyroiditis is stained by anti-human globulin antibodies, indicating presence of globulin in that tissue.‡

Since thyroid sections used in this study fluoresced green in certain areas under ultraviolet light without any staining and since this green was difficult to distinguish from the green staining obtained with the fluorescein label developed by Coons, the newly developed tetramethylrhodamine label was particularly useful. The tetramethylrhodamine fluoresces orange-red rather than the green and can be easily distinguished from background fluorescence.

Materials and methods. The chronic thyroiditis serum used was described previously (10). The diseased thyroid was that obtained from the same patient. Normal serums of blood groups A, B and O were employed as controls. The normal thyroid tissues used as controls were obtained at autopsy. Rabbit antiserum to human globulin was prepared by injecting rabbits with commercial poliomyelitis immune globulin (human) in Freund's adjuvants(11). *Preparation of 3-amino-tetramethylrhodamine.* 4-Nitrophthalic acid was dissolved in excess ammonium hydroxide and reduced by heating at 80° with a solution of sulfur in excess ammonium sulfide. Sulfur was filtered off and aminophthalic acid precipitated with hydrochloric acid. The aminophthalic acid was dissolved in excess aqueous sodium carbonate and acetylated with acetic anhydride until the product was no longer diazotizable. The acetoaminophthalic acid was condensed with excess 3-dimethylaminophenol by heating at 160° to 170° in tetrahydronaphthalene for 5 hours. The solvent was decanted and the residue washed with benzene and dried. The residue was hydrolyzed by boiling for 10 min. with 5 N HCl. The product was diluted with water and neutralized with sodium carbonate, precipitating the dye. The amino dye so obtained was washed with water and dissolved in acetone. Dilution of the filtered acetone solution with water precipitated the dye, aminotetramethylrhodamine, which was then dried and used without further purification or separation into isomers.

Tetramethylrhodamine and fluorescein labelled proteins. Essentially the same procedure described by Coons and Kaplan(12) for conversion of aminofluorescein to the isocyanate by treatment with phosgene and coupling the isocyanate to protein was used for fluorescein and tetramethylrhodamine coupling. The coupled protein solution was dialyzed and was stored in the frozen state. Prior to use, the labelled protein was absorbed with acetone rat liver powder(13). *Staining procedure.* Sections of normal thyroid tissue were cut and dried under a lamp for 10 min. In some cases, the tissue was fixed in acetone, methanol or ethanol for 15 min. at room temperature before drying. A drop of the serum to be tested was placed on the section and incubated for 1 hr at room temperature. The sections were then washed in saline for 10 min. with agitation and incubated similarly with tetramethylrhodamine or fluorescein labelled rabbit antibody to human globulin for 1 hr and washed again. Observations were made with a Reichert fluorescence microscope; the section was mounted in glycerol. Sections of Hashimoto's disease thyroid and lymph nodes were fixed as above or in formalin for 30 min. and stained directly by the rhodamine labelled anti-human globulin antibody.

Results. When normal thyroid obtained at necropsy was employed in the staining procedure, there was specific staining only when the chronic thyroiditis serum was employed. Staining occurred in the colloid region and in the glandular epithelium of the thyroid as

‡ Dr. R. G. White has made somewhat similar observations with fluorescein labelled serum from a Hashimoto's disease patient. Beutner *et al.*(8) also mention similar observations.

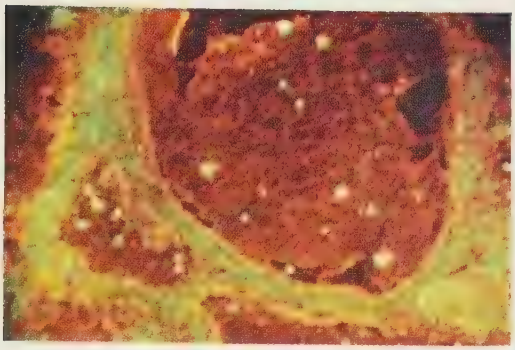

FIG. 1. Normal thyroid treated with chronic thyroiditis serum and subsequently with tetramethylrhodamine labeled rabbit anti-human globulin antibody. Orange fluorescence due to staining was seen in the colloid region of the thyroid and also in the glandular epithelium of the thyroid. Strong green autofluorescence of the stroma was present.

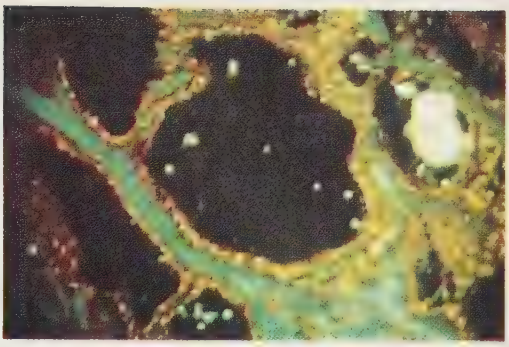

FIG. 2. Normal thyroid treated with normal human serum and subsequently with tetramethylrhodamine labeled rabbit anti-human globulin antibody. No staining was seen in the colloid region—much of the colloid appeared to be washed away. Nonspecific staining occurred in the glandular epithelium about equivalent to controls not treated with human serum prior to staining.

FIG. 3. Normal thyroid treated as in Fig. 1 except that fluorescein rather than tetramethylrhodamine was used as label. It is apparent that the colloid is stained by the fluorescent antibody but the green fluorescence of the unstained stroma does not allow observation of any specific staining in or close to the stroma.

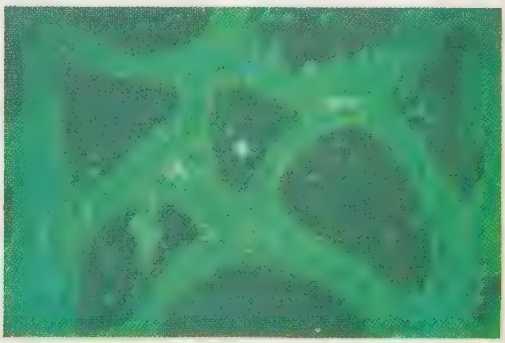

FIG. 4. Same as Fig. 2 except that fluorescein rather than tetramethylrhodamine was used as label. Nonspecific staining of the glandular epithelium is also seen.

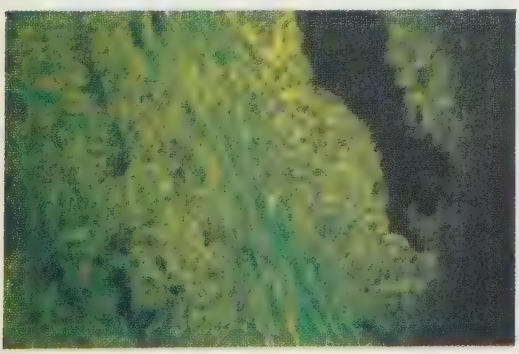

FIG. 5. Chronic thyroiditis thyroid treated with rhodamine labeled rabbit anti-human globulin antibody. Staining was present in the areas of lymphoid cell infiltration, showing the presence of globulin in or around these cells. Strong green-blue autofluorescence was present in the stroma.

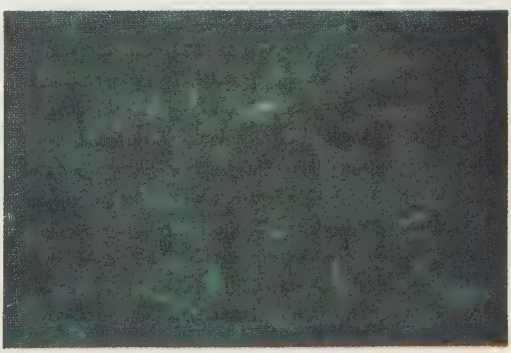

FIG. 6. Normal lymph node treated with tetramethylrhodamine labeled rabbit anti-human globulin antibody. The lymphoid tissue except for occasional weak areas of orange fluorescence was negative.

shown in Fig. 1 where staining was done with the tetramethylrhodamine labelled serum. The staining in the glandular epithelium may have been largely nonspecific since control sections treated with serum of individuals of the several blood groups showed no fluorescence in the colloid but there was staining of the glandular epithelium about equivalent to the staining of control sections which had not been treated with any human serum prior to staining with rabbit anti-human globulin antibody. The colloid appeared to have been leached out (Fig. 2).

The results obtained with the tetramethylrhodamine label (Fig. 1 and 2) were compared with results obtained with the fluorescein label (Fig. 3 and 4) where similar sections treated with chronic thyroiditis serum (Fig. 3) and normal human serum (Fig. 4) prior to staining with fluorescein labelled anti-human globulin antibody are shown. Here, it can be seen that the colloid was stained when chronic thyroiditis serum was used, but much of the other detail was lost because the fluorescence due to fluorescein label was indistinguishable from the background fluorescence of the tissue itself. Differentiation between fluorescent stain and adjacent background fluorescence is only possible when the tetramethylrhodamine stain is used.

In the staining procedures, there was some leaching out of the soluble antigen which gave precipitate with the added chronic thyroiditis serum. No such precipitate was obtained with control serum. Although acetone fixation did not give completely satisfactory results, it appeared better than ethanol, methanol or formalin fixation. The precipitate formed in solution and was removed on subsequent washing. Staining was obtained even when the precipitate formed. This indicates that there was an excess of staining antibody present or that perhaps the components which are being stained in the tissue are of a different antigenic nature than the material which leached out and formed a precipitate. The possibility does exist that in chronic thyroiditis there may first be formed antibodies directed against components other than thyroglobulin and these are the originally cytotoxic antibodies. Antibodies to thyroglobulin may be formed only subsequent to the initial insult by these hypothetical antibodies directed against other components.

It appears from the staining results that antibody prepared in rabbits against the polio globulin contains antibody against the component of chronic thyroiditis serum responsible for precipitation with thyroid extract. It has been shown that this component is associated with the rapidly sedimenting globulin(10). Similar rapidly sedimenting components are known to be constituents of normal serum. Therefore, the chronic thyroiditis serum component must be similar antigenically to normal serum components.

To determine whether human globulin was present in the chronic thyroiditis thyroid, formalin fixed§ diseased thyroid was treated with tetramethylrhodamine labelled rabbit anti-human globulin antiserum for 1 hr. Formalin fixed sections of lymph node were treated simultaneously as control since chronic thyroiditis thyroid is primarily a mass of lymphocytic infiltration. The sections were washed and observed. Good staining was present in the case of the thyroid from the patient with chronic thyroiditis with orange fluorescence being present primarily in the infiltrating lymphocytic cells, showing the presence of globulin in or perhaps around the cells while the stromal elements showed strong green and blue auto-fluorescence (Fig. 5). The lymphoid tissue except for occasional weak areas of orange fluorescence was negative (Fig. 6). This indicates that the infiltrating cells contained globulin and may have been important in the destruction of the thyroid during the course of the disease.

To demonstrate specificity of the staining, thyroid sections from chronic thyroiditis were first incubated with unlabelled anti-human globulin (2 hr), washed briefly and then incubated for 10 min. with tetramethylrhodamine labelled rabbit anti-human globulin. The sections were washed for 5 min. before observation. There was definite inhibition of staining. As controls, similar sections were treated with normal rabbit serum in place of the unlabelled anti-human globulin serum and

§ Similar results were obtained with acetone fixed sections.

showed no inhibition in subsequent staining with the labelled anti-human globulin. This proves that the staining observed originally was due to the reaction of anti-human globulin with human globulins in the section.

Whether the slides were fixed in formalin‖ or acetone or not fixed at all, there was a definite precipitate formation when the unlabelled antihuman gamma globulin was added. The precipitate formed in solution and was removed on subsequent washing. The fact that staining was obtained in the presence of the precipitate forming in the suspension would indicate here also that there was excess antibody present and perhaps that the components which are being stained in the tissue may be of a different nature than the material precipitating.

Summary. 1) A new fluorescent label, tetramethylrhodamine isocyanate, is described and its use demonstrated in the immunohistochemical fluorescence technic. The label fluoresces orange and is of particular value for staining tissues which show a green autofluorescence similar to fluorescein fluorescence even when unstained. 2) Serum from a patient with chronic thyroiditis combined with normal thyroid tissue in the colloid region and perhaps in the glandular epithelium, while normal human serum showed no such staining. Thyroid from a patient with chronic thyroiditis, infiltrated with lymphocytic cells, was stained by rabbit antibody to human globulin in the region occupied by the infiltrating cells. Normal lymph node showed no such staining. This indicates that the infiltrating cells contained human globulin and may have been important in the destruction of the thyroid during the course of the disease.

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‖ The fixation process did not render the antigens completely insoluble.

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Role of 11β-Hydroxyprogesterone as Intermediary in Biosynthesis of Cortisol and Corticosterone.* (23823)

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There is considerable evidence that production of 17α-hydroxycorticosterone (cortisol) and corticosterone from cholesterol in the adrenal cortex proceeds *via* the reaction scheme illustrated in Fig. 1(1,2). The indicated sequence of hydroxylations of progesterone, shown by solid arrows, has been re-

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† Portion of thesis submitted in partial fulfilment of requirements for degree of Doctor of Philosophy, Dept. of Physiology, Boston University Medical School.

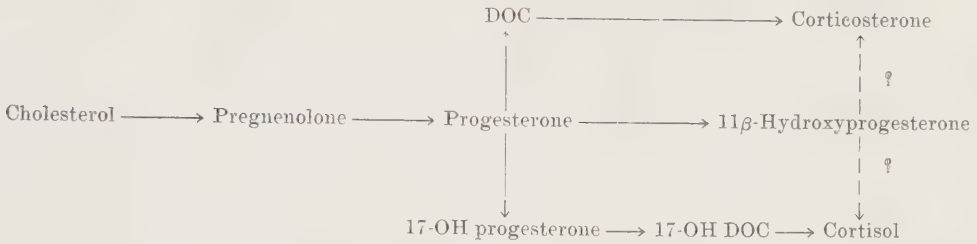

FIG. 1. Corticosteroidogenic Sequence.

garded as obligatory, and was postulated as the major route of biosynthesis of these corticoids. As indicated in Fig. 1, there is uncertainty as to the possible role of 11β-hydroxyprogesterone (HP) as an intermediary in this sequence. Although progesterone is converted in adrenal tissue to 11β-HP, the latter was originally considered not to be a *major* intermediary on the basis of adrenal perfusion experiments wherein 11β-HP failed to give rise to significant amounts of cortisol or corticosterone(3). However, these perfusion data do not permit a definitive conclusion, since adequate transfer of 11β-HP from the perfusate into the cells may not have occurred, whereas 11β-HP formed intracellularly might serve as an intermediary. The more recent findings that adrenal mitochondrial systems(4,5) convert progesterone to 11β-HP in good yield have served to reopen consideration of 11β-HP as a possible intermediary in corticosteroidogenesis.

To eliminate the influences of permeability barriers inherent in the perfusion technic, we have employed bovine adrenocortical homogenates, which synthesize corticoids from endogenous precursors, to reinvestigate the role of 11β-HP as an intermediary in corticoid biosynthesis. Tracer amounts of 11β-HP-4-C^{14} were incubated in homogenates prepared in various media, and the degree of incorporation into the cortisol and corticosterone formed was measured relative to that of comparable experiments with tracer amounts of progesterone-C^{14} (labelled either at C-4 or C-21) and DOC-21-C^{14}. The results of these studies demonstrate that while 11β-HP may be converted to both cortisol and corticosterone, comparison of the rates of conversion relative to progesterone and DOC indicate that in bovine adrenal tissue, 11β-HP is not a *major* intermediary in the biosynthesis of cortisol or corticosterone from progesterone.

Methods. A. *Preparation of homogenates.* Bovine adrenal cortex tissue was homogenized in each of the following media, using procedures previously described in detail(6): (a) 0.025M sucrose, (b) 0.154M KCl and (c) 0.154M NaCl. All media were buffered with M/150 Sorensen's phosphate (pH 7.2) and supplemented with niacinamide (.005M), sodium fumarate (.005M), $MgSO_4$ (.005M), adenosine triphosphate (ATP) (.001M) and diphosphopyridine nucleotide (DPN) (.0005M). B. *Steroid substrates.* The C^{14}-progesterone (S.A. 1.60 x 10^6 counts/min./mg) and DOC-21-C^{14} (S.A. 1.55 x 10^6 counts/min./mg) employed in these studies were highly purified preparations and regarded as radiochemically homogenous on the basis of our previous studies(6). The 11β-HP-4-C^{14} employed was a highly purified crystalline sample, kindly supplied by Dr. Harold Levy of these institutions, and was derived from bovine adrenal perfusion of progesterone-4-C^{14}. The sample had been fully characterized by Dr. Levy on the basis of standard organic technics as well as by I.R. Upon subjecting this sample to paper chromatography using a ligroin-toluene (1:1)/propylene glycol system, a modification of Zaffaroni(7), a single steroid zone was detected. Upon subsequent chromatography on silica gel columns, eluting with large volumes of a benzene-ethyl acetate (10:1) mixture, a single sharp symmetrical peak of radioactivity was observed which corresponded to the 11β-HP and contained more than 99% of the radioactivity. The 11β-HP employed had an original specific activity (S.A.) of 6.58 x 10^6 c./m./mg and a portion of this sample was diluted to S.A. of 1.55 x 10^6

c./m./mg. C. *Incubation conditions.* Homogenate aliquots corresponding to 35 to 100 g tissue were added to reaction vessels containing the radioactive steroid suspended in 0.3 ml propylene glycol, and were incubated for 2 hr with shaking (gas phase, 100% O_2). The radiosteroids were present in the various experiments in ratios ranging from 1.1 to 4.6 μM per 100 g equivalents of tissue (equivalent to 400 ml homogenate). After incubation the reactions were halted by the addition of 2 volumes of acetone. In each experiment aliquots of the same homogenate extracted without incubation served to measure the cortisol and corticosterone originally present in the homogenate, and thus permitted measurement of the net synthesis of these corticoids during the 2 hr period of incubation. D. *Extraction and isolation of corticoids.* The procedure employed has been previously described in detail(6). Briefly, this consisted of exhaustive extraction of homogenates with acetone and ethyl acetate, removal of the bulk of phospholipides by acetone-$MgCl_2$ precipitation in the cold, followed by fractionation on silica gel columns. The radioactive fractions eluted from the column were then divided into 2 equal portions, one of which was reserved for the procedures described in "E". The other portion was subjected to paper chromatography and the corticosterone and cortisol zones were cut out, eluted and rechromatographed. These steroids were then acetylated and subjected to 2 successive paper chromatograms on systems suitable for the monoacetates. At this stage of fractionation the corticosterone acetate and cortisol acetate were considered to be sufficiently pure to provide meaningful results concerning incorporation of C^{14}-substrates. The samples were counted (infinite thinness, using a flow-gas counter) and then quantitatively estimated using blue tetrazolium in a modified Mader-Buck procedure. E. *Radiochemical homogeneity of the corticosteroids.* This was assessed by determining the S.A. of the corticosteroids at various stages of purification. For this purpose, the portions of extracts remaining (50%) were appropriately pooled as described in the discussion of results. The chromatography of the steroid alcohols and monoacetates, as described above, was repeated on these pooled samples and the following additional derivatives were prepared: for corticosterone, (a) oxidation of the corticosterone-21-acetate to 11-dehydrocorticosterone-21-acetate *via* CrO_3(8), and (b) saponification of "a" to 11-dehydrocorticosterone by means of methanolic $KHCO_3$(9); for cortisol, (a) the cortisol-21-acetate was oxidized to cortisone acetate(8), and on the 11β-HP samples adrenosterone was prepared from the cortisol(10). These derivatives were chromatographed in appropriate paper systems (7), eluted, and S.A. determined.

Results obtained upon incubation of C^{14}-steroid substrates with bovine adrenal homogenates are shown in Table I. In Ia, aliquots of a sucrose homogenate were incubated with an equal number of counts (2.4×10^6 c./m.) of C^{14}-11β-HP and C^{14}-DOC; results obtained with C^{14}-progesterone in identical sucrose homogenates in two other experiments are shown in Ib for comparison. It will be seen that whereas the incorporation of 11β-HP-4-C^{14} into cortisol or corticosterone could not be detected, incorporation of C^{14}-DOC into corticosterone and C^{14}-progesterone into both corticosteroids is readily demonstrable. In I, the total number of μM of C^{14}-steroid substrate incubated were different; the 11β-HP had a higher S.A. and was present in lowest concentration. In II, C^{14}-11β-HP, C^{14}-progesterone and C^{14}-DOC (all at a concentration of 2.8-2.9 μM per 100 g tissue equivalent), were incubated in various homogenates all derived from the same pool of adrenocortical brei. In this experiment, C^{14}-11β-HP was incorporated into both cortisol and corticosterone in all media. However, C^{14}-11β-HP is incorporated into the corticosterone synthesized to a lesser extent than either C^{14}-progesterone or C^{14}-DOC, independently of the type of media employed. With regard to the conversion of 11β-HP to cortisol relative to progesterone, the results differ depending upon the media used. In sucrose homogenates, wherein C^{14}-progesterone conversion to cortisol occurs at the greatest rate, progesterone is superior to 11β-HP in confirmation of the results obtained in I. When homogenates are prepared with ionic media, C^{14}-progester-

TABLE I. Products Isolated from Bovine Adrenocortical Homogenates Prepared in Various Media and Incubated with C^{14}-Substrates.

	C^{14}-substrate*	Tissue wt (wet), g	C^{14}-substrate added/100 g tissue μM	Cts/min. (× 10^6)	Medium	Net steroid production (mg/100 g/2 hr) B†	F‡	C^{14}-substrate conversion (μg/100 g/2 hr) B	F	S.A. (cts/min./μg) B	F	S.A. B / S.A. F
Ia	11β-HP-4-C^{14}	44	1.1	2.47	Sucrose	1.89	.71	<0.2	<0.2			
	DOC-21-C^{14}	"	4.6	2.36	"	2.50	.76	415	<0.5	257		
Ib	Progesterone-21-C^{14}	100	1.7	.87	"	2.12	1.52	76	27	57	28	2.0
	Progesterone-4-C^{14}	60	1.6	.80	"	2.60	2.58	123	52	76	32	2.3
II	11β-HP-4-C^{14}	35	2.8	1.47	"	2.39	.87	21	4	14	7	1.9
					KCl	.69	1.16	13	7	29	9	3.4
					NaCl	.97	.70	29	7	46	16	3.0
	Progesterone-4-C^{14}	"	2.9	1.47	Sucrose	1.31	.89	51	18	62	33	1.9
					KCl	.68	.82	34	2	80	5	17.0
					NaCl	1.18	.77	81	4	110	8	13.6
	DOC-21-C^{14}	"	2.8	1.47	Sucrose	1.98	.76	137	<0.5	107		
					KCl	1.04	1.09	45	"	67		
					NaCl	1.02	.82	72	"	110		
III	11β-HP-4-C^{14}	46	3.8	1.97	KCl	.88	.74	21	9.6	37	20	1.9
	Progesterone-21-C^{14}	"	4.1	2.08	"	2.09	1.16	182	9.5	139	13	10.7
	DOC-21-C^{14}	"	4.5	2.36	"	1.52	1.20	145	<0.5	148		

* Specific activity (S.A.), cts/min./μg: 11β-HP, in I 6580, in II and III 1550; progesterone 1600; DOC 1550.

† B = corticosterone. ‡ F = cortisol.

one conversion to cortisol is markedly reduced, whereas that of 11β-HP is little affected; the net result is that under these conditions C^{14}-11β-HP is incorporated into the cortisol formed at an equal or greater extent than C^{14}-progesterone. To check this result, in III aliquots of an homogenate prepared in KCl were incubated with C^{14}-11β-HP, C^{14}-progesterone and C^{14}-DOC. The results with regard to incorporation into the cortisol synthesized were essentially similar to those obtained in II.

Table I shows incorporation of C^{14}-steroids into corticosteroids, based upon radioactivity present following chromatography of cortisol and corticosterone as the free steroid alcohol and as the monoacetate. To determine whether the corticosteroids isolated from 11β-HP incubations were radiochemically homogeneous at this stage of purification, 50% portions of the original column eluates of the 11β-HP incubations were combined to obtain separate pools of the ionic and sucrose incubations. For comparison the eluates from C^{14}-progesterone incubations were likewise pooled. These pooled samples were chromatographed, derivatives prepared, and rechromatographed at each stage. The specific activities of these samples at various stages are shown in Table II, from which it will be seen that the cortisol and corticosterone from the sucrose incubations may be considered radiochemically homogeneous at the acetate stage. However, the homogeneity of the products of the incubations in ionic media remains questionable despite the fairly rigorous purification procedures.

Discussion. It seems clear from the S.A. data in Table II, that 11β-HP-4-C^{14} can be converted to both cortisol and corticosterone in the systems employed. The question then arises whether 11β-HP is a major intermediary in the reaction sequence, progesterone to cortisol or corticosterone. It is a biochemical maxim that in any reaction sequence a postulated intermediary reaction should proceed at

TABLE II. Specific Activities of Products Derived from C^{14}-11β-HP and Progesterone at Successive Stages of Purification.

Products derived from C^{14}-substrates	S.A. counts/min./μg			
	Sucrose incubations		Ionic incubations*	
	11β-HP	Progesterone	11β-HP	Progesterone
Cortisol†	35	39	27	23
" ‡	18	31	20	12
Cortisol-21-acetate†	10	36	11	7
Idem ‡	7	36	10	6
Cortisone-21-acetate	5	32	8	7
Adrenosterone	5		4	
Corticosterone†	49	64	154	154
" ‡	46	68	140	136
Corticosterone-21-acetate†	11	67	48	126
Idem ‡	11	53	48	132
11-dehydrocorticosterone-21-acetate	8	59	36	108
11-dehydrocorticosterone	13	55	33	99

* Products from KCl and NaCl incubations combined.

† After 1st chromatogram.

‡ " 2nd " .

a rate equal to (if not greater than) the rate of the overall process. From this point of view, it is clear that 11β-HP cannot be the major intermediary in the conversion of progesterone to corticosterone, since under all conditions studied, the conversion rates were less than those obtained with progesterone or the alternative intermediary, DOC. On the other hand, DOC is converted to corticosterone at rates equal to or exceeding those of progesterone.

The role of 11β-HP as an intermediary in cortisol production is more difficult to interpret because the incorporation of C^{14}-progesterone into cortisol is influenced by the nature of the homogenate preparation. In sucrose homogenates, where C^{14}-progesterone is incorporated into cortisol at the highest rate, C^{14}-11β-HP is definitely inferior. When ionic media are used, C^{14}-progesterone apparently does not mix well with endogenous progesterone at the site of the 17-hydroxylation step, since despite the fact that endogenous cortisol production is not diminished the incorporation of C^{14}-progesterone is markedly reduced. This is also evident from the fact that with C^{14}-progesterone the ratio of S.A.'s of corticosterone/cortisol is increased from about 2 in sucrose to 11-17 in ionic media.

To determine the significance of 11β-HP as cortisol intermediary, one must decide which type of homogenate most nearly exhibits the pattern of corticosteroidogenesis observed in the intact adrenocortical cell. Fortunately, data are available to help decide between these alternatives. In experiments wherein C^{14}-progesterone was perfused through bovine adrenals(11), it was found that the S.A. ratio of the products formed (S.A. corticosterone/S.A. cortisol), was 1.5; this is of the same order of magnitude as in the sucrose homogenates. Thus it would appear that the components involved are organized in sucrose homogenates in a manner that is similar to the arrangement in intact cells of the perfused gland, whereas the conditions in the homogenates prepared with ionic media are "abnormal" relative to intact cells. From these considerations, the conclusion emerges that 11β-HP, while capable of being converted to cortisol by adrenal enzymes, is *not* a major intermediary in the formation of cortisol from progesterone in the cow gland.

Summary. Trace amounts of C^{14}-11β-hydroxyprogesterone, C^{14}-progesterone, and C^{14}-DOC were incubated with homogenates of adrenocortical tissue prepared in various media. These homogenates, which synthesize corticoids from endogenous precursors, hydroxylated C^{14}-11β-hydroxyprogesterone at both the C-21 and C-17 positions, producing cortisol and corticosterone. However, the limited extent of these conversions relative to that of known precursors (progesterone and DOC) indicates that 11β-hydroxyprogesterone is not a major intermediary in the biosynthesis of these corticoids from progesterone.

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Anti-hypertensive Action of 17-Imidazolyl-steroids in the Rat. (23824)

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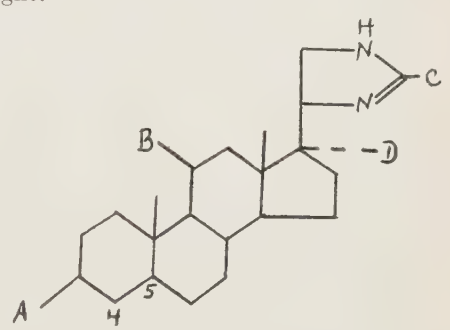
Metacorticoid hypertension is a self-sustaining cardiovascular disease attained in rats some 3 months after the implantation of a desoxycorticosterone acetate (DCA) pellet and the substitution of saline for drinking water. In many respects, this type of hypertension appears similar to essential hypertension in man(1) and responds to treatment with various drugs active in the human disease(2,3). In addition, certain steroids(4-6) and steroid-analogs(7) have been reported to exert transient depressor effects in metacorticoid hypertension. The present report describes similar activity in a new series of compounds, 17-imidazolyl-androstenes(8).

Acute experiments. This study utilized 70 male, Sprague-Dawley rats that had received subcutaneously a 40 mg wax pellet containing 20 mg DCA at least 3 months previously and that had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. Saline and food were not removed during the course of the experiment. The systolic blood pressures of treated rats were estimated without anesthesia or heating using a photoelectric tensometer(9) before and 2, 4 and 6 hours after treatment, and were compared to simultaneous readings from propylene glycol-injected controls. Pressure readings on individual rats were made in ignorance of the injection the rat had received and of the previous pressure reading. Each test usually consisted of 4 treated and 4 control rats run simultaneously by one person on the same tensometer in a darkened room, with injections made by a different person. In order to minimize leakage, subcutaneous injections were always made at least 60 mm from the site of skin puncture with a 20 g hypodermic needle. The following 6 steroids were injected in a total of 35 rats as 1% solutions in propylene glycol at a dosage of 20 mg/kg body weight:



	A	B	C	D	Δ
SC-4200	OH	H	H	H	5
-4225	O	H	CH_3	H	4
-4577	O	H	H	OH	4
-6243	O	H	H	H	4
-6255	OH	H	CH_3	H	5
-6307	O	O	H	OH	4

Table I lists the mean pretreatment blood pressures and the changes observed after treatment with the 6 steroids; all control data are pooled. The probability values were read from Fisher's Table I(10) after applying the rank-sum test(11) to the blood pressure changes. On this basis, significant hypotensive effects were produced only by SC-4200

TABLE I. Blood Pressure Effects of 17-imidazolyl-steroids.

Compound	No. rats	B.P. before inj.	Change in B.P. after inj. 2 hr	4 hr	6 hr
SC-4200	8	188	−22*	−27†	−34‡
-6255	4	192	− 6	− 4	− 7
-6243	4	187	−13	−15	−14
-4225	7	190	−11	−15	−14
-4577	8	187	−26†	−32†	−23†
6307	4	184	− 2	−10	−20
Pooled controls	35	186	− 2	− 1	− 1

* $P = 0.06$.
† $P < 0.01$.
‡ $P < 0.001$ that treated = controls.

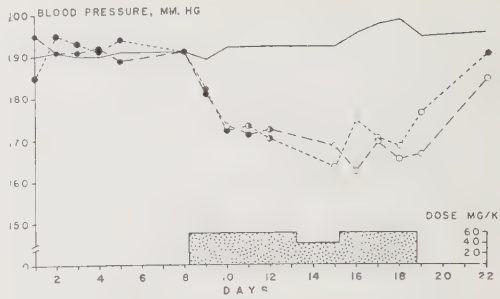


FIG. 1. Effect of SC-4200 (– – –), SC-4577 (----), and placebo (——) on blood pressure of metacorticoid hypertensive rats. Six rats per group unless otherwise noted. Significance of changes in blood pressure, compared to those of controls: $P > 0.05$ (solid circles), < 0.05 (halved circles), < 0.01 (open circles).

and SC-4577. From consideration of the structures of the compounds, it appears that the methylation of the imidazole ring of SC-4200 decreases activity (SC-6255), while the activity of SC-4577 is reduced by 11-oxidation (SC-6307) or 17-dehydroxylation (SC-6243, SC-4225).

Chronic experiments. Eighteen additional metacorticoid rats were divided into 3 equal groups for the chronic administration of the 2 active compounds, SC-4200 and SC-4577, plus a corn oil-placebo. The experimental conditions and technics were in general the same as in the acute experiments. Six control blood pressure readings were taken over the 8 days before injection; individual rat blood pressures varied over a 26 mm range during this period, at absolute levels over 178 mm. Beginning the 8th day, while the controls received equivalent volumes (2-3 ml/kg/day) of the solvent, the treated groups received either SC-4200 or SC-4577 (2% in corn oil, subcutaneously) in the following doses: 20 mg/kg t.i.d. x 5 days, then 40 mg/kg q.d. x 2 days, and finally 20 mg/kg t.i.d. x 4 days; this was a total dosage of 620 mg/kg in 11 days. Blood pressures were taken 17-18 hours after the preceding injection (Fig. 1). All surviving rats were killed 4 days after the end of the treatment period.

Chronic treatment with either compound significantly lowered blood pressure and there was no apparent difference in potency. Both compounds exhibited a latent period of 3-4 days at the start of treatment and a fairly rapid (4-day) recovery of hypertensive levels after cessation of treatment. One of the SC-4577 rats died after 5 days of treatment; he was found to have severe periarteritis nodosa, nephrosclerosis, and infected lungs and pancreas. Most of the remaining rats had pneumonitis and subcutaneous residues of the compound, but no gross evidence of toxicity was found in any of the treated rats. Moreover, there were no significant losses of body weight and the relative organ weights of the 3 groups did not differ significantly (Table II). The mechanism of hypotensive action of these 2 compounds is unknown, but is not correlated with any glucocorticoid, androgenic, or anabolic activity, as determined in our laboratories by Dr. F. J. Saunders.

Summary. Six imidazolyl-steroids were tested for depressor activity in metacorticoid hypertensive rats. Two compounds were active acutely and chronically: 17β-(4[5]-imidazolyl)-5-androsten-3β-ol and 17α-hydroxy-

TABLE II. Organ Weights at Necropsy, %.*

Group	No. rats	Wt, g	△ Wt, g	B.P., mm	Adrenals	Thymus	Heart	Kidneys	Testes
SC-4200	6	413	+ 7	184	.018	.102	.388	.919	.865
SC-4577	5	391	+ 3	187	.016	.087	.411	.899	.898
Placebo	6	380	+18	197	.019	.098	.384	.830	.915

* $P > 0.05$ that treated = controls.

17(4|5]-imidazolyl)-4-androsten-3-one. Activity was apparently reduced by methylation of the imidazole ring of the former and by 11-oxidation or 17-dehydroxylation of the latter.

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Regeneration of Liver in Lysine-Deficient, Partially Hepatectomized Rats.* (23825)

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Coincident with starvation or maintenance on nitrogen-free diet, there occurs a rapid and extensive loss of nitrogen from liver of rats (1,2). Upon realimentation with protein of adequate nutritional quality, the nitrogen content of liver returns to normal levels in a few days. This accumulation of nitrogen during realimentation of protein-depleted rats has been used for nutritional evaluation of proteins(1,3), of amino acid mixtures and of protein hydrolyzates(4). Similarly, after partial hepatectomy of rats, proliferation of the remaining liver occurs to some extent when they are post-operatively starved(5) or fed protein-free diet(6), and progressively more so as the diet furnishes increased amounts of proteins(6) of adequate nutritional quality(7).

It has been clearly demonstrated that adult rats fed a lysine-deficient amino acid mixture incur loss of appetite and body weight(8), negative nitrogen balance(9) and marked decrease of total circulating serum protein(10). In view of the importance of liver for formation of plasma proteins(11), abnormalities in composition of liver induced by imbalance of dietary amino acids or proteins(12) and increasing evidence that lysine can be a limiting factor in the nutritive value of some diets (13), it was of interest to investigate the effect of lysine deficiency on regeneration of liver.

Methods. Mature female rats of hooded strain, maintained in our colony for many years, weighing initially about 200 g were housed individually in cages with screen bottoms. They had access to food and water *ad libitum*. Records were kept of food consumption and each animal received a daily allotment of the ration (Table I) estimated to exceed slightly the

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TABLE I. Composition of Rations.

Component	Complete ration (g)
Amino acid mixture*	223.2
Sucrose and B vit. mixture†	20.
Salt mixture†	40.
Wheat germ oil	20.
Corn oil + vit. A and D_3†	10.
Sucrose	686.8
	1000.

* The mixture for the complete ration contained: L-arginine hydrochloride 12.96, L-histidine hydrochloride 4.75, L-lysine hydrochloride 13.79, L-tyrosine 6.56, DL-tryptophan 5.12, DL-phenylalanine 18.56, L-cystine 3.68, DL-methionine 10.58, DL-threonine 16.00, L-leucine 15.04, DL-isoleucine 22.08, DL-valine 23.68, L-glutamic acid 29.44, DL-aspartic acid 26.24, glycine 14.72 g. In the mixture for the lysine-deficient ration two molar equivalents of L-glutamic acid replaced the lysine.

† Composition given in reference 21.

intake. The amino acid mixture contained the L-amino acids in essentially the proportions found in whole egg(14) except that glycine was substituted for L-serine and L-glutamic acid for L-proline. In the lysine-deficient ration, L-glutamic acid was substituted in 2 molar proportions for L-lysine contained in the complete amino acid mixture. About 10 rats were in each of 7 groups. Group A was fed the complete ration for 14 days and then killed to determine the ratio of liver to body weights, a ratio used to estimate amount of liver not removed from Groups C and E. Groups B, C and F received the complete ration for 22 days; Groups D, E and G received the lysine-deficient ration for 22 days. Animals of Group C and E were partially hepatectomized on the 15th day, under ether anesthesia, by the method of Ralli and Dumm (15), a procedure by which an estimated 54.9 $\pm$ 3.67%‡ and 55.8 $\pm$ 3.0% respectively, of the liver were removed. On the same day groups B and D were subjected to sham operation which included anesthesia, exteriorization of the liver through the incision, replacement of liver and suturing. The rats were killed 8 days after operation when regeneration of liver can be essentially complete(6). Groups F and G served as non-operated controls. All animals were killed with chloroform. Whole livers, like the lobes which were removed by partial hepatectomy, were immediately weighed, partially dried at 60°C, then ground in glass mortar. The ground livers were analyzed for residual moisture by drying *in vacuo* at 90°C for 8 hours and for total lipids(16) and total N(17).

Results. In spite of lysine deficiency, rats in Group E were able to regenerate a major portion of the liver during 8 days, when their livers had attained 90.7 $\pm$ 3.4% of estimated fresh weight before hepatectomy; in Group C which consumed the complete ration the corresponding figure was 89.9 $\pm$ 2.1%.

As shown in Table II this increase in weight of liver, after partial hepatectomy, was accompanied by some changes in composition. In the lysine-deficient Group E, there was a significant increase in % moisture ($P = < 0.01$)§ and in lipids ($P = <0.01$) and a decrease in % N ($P = <0.01$). In Group C lipid content increased ($P = <0.01$) and the N content decreased ($P = <0.01$) while the change in % moisture was not significant. Considering the estimated amount of N remaining after partial hepatectomy and the final N content of livers, lysine-deficient rats deposited during recovery a mean of 51.7 $\pm$ 10.1 mg of N compared to 77.6 $\pm$ 16.1 mg for rats fed the complete ration. Calculated on a weight basis these values are 31.6 mg and 38.7 mg of nitrogen/100 g of final body weight respectively; the difference is not statistically significant. Formation of new liver tissue and deposition of N therein was accomplished by lysine-deficient rats in spite of the fact that during the preceding 2 weeks they had been in negative N balance, as evidenced by a loss of body weight, and that this condition continued during the period of regeneration.

Changes in composition of liver were partly due to surgical procedure. Comparison of sham operated groups B and D with the unoperated groups F and G respectively, revealed that the former had a significantly greater ($P = <0.01$) liver size with increased concentration of lipid ($P = <0.05$) than the rats fed either lysine-deficient or the complete ration ($P = <0.01$). Concentration of N was lower ($P = <0.05$) in livers of the sham

‡ Standard error of mean.

§ P = probability that the difference is due to chance.

TABLE II. Composition of Livers.

Group	Lysine in diet, %	Operation*	No. of rats	Wt change, g	Liver				
					Dry wt, g	% of body wt	Moisture, %	Lipid, % of dry wt	Nitrogen, % of dry wt
B	1.38	Sham	9	+ .9 ± 2.0†	2.4 ±.04	4.0 ±.07	70.5 ± .36	23.7 ±1.37	10.1 ±.10
D	0	"	10	−26.2 ± 2.3	2.2 ±.12	4.2 ±.17	71.0 ±1.85	29.0 ±2.55	9.6 ±.41
C	1.38	At P.H.	11	− 6.8 ± 3.6			70.1 ± .43	19.3 ± .61	11.0 ±.34
		When killed	11	− 2.6 ± 4.3	2.0 ±.15	3.4 ±.10	71.1 ±1.20	23.1 ± .92	9.7 ±.18
E	0	At P.H.	9	−25.7 ± 1.3			70.5 ± .50	19.6 ± .73	10.4 ±.23
		When killed	9	−29.6 ± 1.6	1.5 ±.18	3.4 ±.18	72.7 ± .51	25.0 ± .83	9.4 ±.21
F	1.38	Non-op.	10	+9.6 ± 2.8	2.1 ±.07	3.5 ±.11	71.2 ± .35	19.0 ± .41	10.8 ±.27
G	0	"	10	−29.6 ± .90	1.6 ±.04	3.2 ±.06	70.9 ± .21	19.9 ± .66	10.2 ±.12

* Sham = sham operation; P.H. = partial hepatectomy; Non-op. = non-operated.
† Stand. error of mean.

operated animals (Group B) fed the complete diet than in livers of unoperated controls (Group F). In contrast, lysine deficiency had no effect on concentration of liver lipids, either after 14 days (compare Groups C and E) or 22 days (compare Groups F and G) of feeding. It did, however, cause a marked decrease ($P = <0.05$) in concentration of N in livers of non-operated control group but not in sham operated or partially hepatectomized groups.

Lysine deficiency caused a marked loss in body weight as would be expected from the decreased food consumption which was, for instance, a mean of 175 g/rat for the 22-day period in Group G, compared to a mean of 224 g in Group F. In lysine-deficient animals the loss of weight was not further accentuated by partial hepatectomy or sham operation, but in rats fed the complete diet the body weight was suppressed by these procedures.

The incisions made during hepatectomy or sham operation of normal animals healed more rapidly than those made into the lysine-deficient rats. The latter chewed on sutures and skin which often left raw areas. This was not observed in rats fed the complete ration. Impaired healing of wounds in protein-deficient rats has previously been reported(18).

The results of these experiments illustrate strikingly that, while the organism as a whole requires a dietary supply of all "essential" amino acids, deposition of nitrogenous compounds and, presumably, the net accumulation or synthesis of proteins can occur in individual tissues of rats fed a lysine-deficient ration. Under these conditions, the incomplete dietary supply of amino acids must have been locally balanced by amino acids which came from degradation of proteins in other, perhaps less vital tissues. Proliferating liver, in this instance, has what others(19) referred to as "priority in utilization of limiting amino acids." Mitchell(20) pointed to a somewhat analogous situation in which "depression in the output of endogenous nitrogen induced by ingestion of methionine may be due to the effect of this amino acid in arresting the raiding of protoplasmic tissues to secure cysteine for hair growth." The preferential synthesis of milk proteins in the mammary gland of animals which are in negative nitrogen balance, is another example of ability of the organism to control protein synthesis, to some extent at least, according to specific localized needs.

Summary. Rats maintained for 2 weeks on a lysine-deficient ration and then partially hepatectomized were able to regenerate their livers in spite of continued consumption of a lysine-deficient diet.

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Comparative Uptake of Calcium-45 and Strontium-90 by Wild and Lordotic Guppies.* (23826)

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Investigations concerning metabolism of bone and mineralized tissues have been hampered by lack of suitable experimental animals. Recently, a naturally-occurring mutation in the guppy, *Lebistes reticulatus* (Peters) has been described which forms bone of greater density and higher calcium content (1). The mutation, a single recessive autosomal character, displays marked dorso ventral and lateral curvature of spinal column which has been called "hunchback"(2,3) or "lordosis"(4). Harrison(5) analyzed 3 animals and found that total body calcium is higher in the mutant fish(5). Rosenthal(1) demonstrated that calcium is increased in lordotic spines while muscle calcium concentration is not altered. The calcium:phosphorus ratios of lordotic bone is also greater since the phosphorus content of bones from both strains of fish is the same(1). These data strongly suggest a specific alteration of calcium metabolism is osseous tissues from lordotic animals.

A series of studies with radioactive calcium-45 and strontium-90 was undertaken in order to determine if the metabolic defect leading to an accumulation of calcium in osseous tissues of the lordotic guppy was due to alterations in the rate of incorporation and/or turnover of mineral elements.

Materials and methods. Wild type guppies were obtained from commercial sources and lordotic guppies were raised in this laboratory, (6). The general experimental design, isotope technics and analytical methods have been previously published in detail(1,7-9). In the present comparative studies with wild

* Neutralizing stones were analyzed for calcium and strontium by Crippen and Ehrlich Laboratories, Baltimore, Md. We are grateful for the spectrographic strontium analysis of bone ash performed through the courtesy of Dr. Karl K. Turekian, Yale University. This investigation was performed under contract with the U. S. Atomic Energy Comm.

TABLE I. Uptake of Calcium-45 by the Total Body of Wild-Type and Lordotic Guppies.

Water bath activity, cpm/ml	Wild-type	Lordotic	T	P
	cpm/100 mg fish/day ± S.E.			
1.79×10^2	38.3± 5.5(6)*	27.9± 2.3(5)	1.84	<.10
3.36×10^3	383 ± 27 (3)	333 ± 40 (2)	2.71	<.05
2.77×10^5	27500 ±2530 (5)	17800 ±1550 (5)	4.02	<.01
1.04×10^6	57600 ±3750 (5)	45100 ±6700 (5)	2.54	<.02
	cpm/mg calcium/day ± S.E.			
1.79×10^2	35.9± 5.0(6)	20.5± 1.8(5)	2.89	<.02
3.36×10^3	336 ± 24 (3)	247 ± 3.0(2)	2.87	<.05
2.77×10^5	24100 ±2440 (5)	13200 ±1560 (5)	5.69	<.01
1.04×10^6	50500 ±3270 (5)	33400 ±4050 (5)	5.09	<.01

* No. of animals analyzed in parentheses.

type and lordotic guppies, male fishes of both strains were placed in the same aquarium and treated in an identical manner. Although tap water aged for 24 hours was used in most instances, conditioned water obtained from stock aquaria was used on occasion. Stock aquaria contained neutralizing stones composed of calcium and strontium salts. Spectrographic analysis of the stones indicated a composition of 64.8% CaO and 27.3% SrO. Since tap water in this area contains 23 ppm calcium and less than 1 ppm strontium(10), it was possible to estimate calcium and strontium content of conditioned water from oxalate precipitable material and composition of neutralizing stones.

Results. Calcium concentration of the body of 56 male lordotic guppies and of 76 wild-type males was 1.35 ± 0.03 (S.E.) and 1.14 ± 0.02 (S.E.) % of wet weight respectively, (t for difference = 12.12, P = < 0.01). Although, whole, fat-free spinal columns from lordotic female guppies contain significantly greater quantities of calcium than spinal columns from wild-type fish, spectrographic analysis of 4 pooled samples consisting of spines from 3 female fish indicated strontium concentrations of 0.028% for wild-type and 0.026% for lordotic bone. It is apparent that lordotic mutation is associated specifically with an alteration of calcium metabolism.

Rate of uptake of calcium-45 from tap water by total body is significantly lower for lordotic guppies at all water activities studied (Table I). The lower rate of uptake becomes even more significant when the data are expressed in terms of body calcium content, since lordotic guppy contains more calcium/unit weight than the wild type. When calcium chloride is added to tap water to bring the calcium content to 275 ppm (Table II), this difference is no longer apparent. However, in conditioned water containing intermediate quantities of salts (75 ppm calcium and 20 ppm strontium), the rate of incorporation of calcium-45 by the body of lordotic guppies remains significantly lower than the rate of wild-type guppies. It may be observed, as reported previously(7), that rate

TABLE II. Uptake and Spine:Body Distribution of Calcium-45 for Wild-Type and Lordotic Male Guppies.

Water*	Body activity ± S.E.		Spine:Body ± S.E.	
	Wild-type	Lordotic	Wild-type	Lordotic
	10^3 cpm/100 mg/day			
Tap H_2O	65.2 ± 4.1 (6)†	50.2 ± 6.1 (7)†	1.33 ± .12 (6)‡	2.18 ± .28 (7)‡
Cond. H_2O—{75 ppm Ca, 20 ppm Sr}	27.5 ± 2.5 (5)§	17.8 ± 1.5 (5)§	1.62 ± .04 (5)§	2.25 ± .16 (5)§
Tap H_2O + 275 ppm Ca	7.35± .43(6)	8.20± .86(6)	2.14 ± .53 (7)	1.79 ± .24 (6)

* Water activity ranged between 2.2×10^5 and 2.8×10^5 cpm/ml water.
† P = <0.05. ‡ P = <0.02. § P = <0.01. All other values not significantly different.
No. of fish analyzed in parentheses.

TABLE III. Rate of Turnover of Incorporated Calcium-45 by Wild-Type and Lordotic Male Guppies.

Organ	Days turnover	Turnover rate* ± S.E. Wild-type	%	Lordotic	%
Carcass	0	2.66 ± .25 (5)†	100	1.72 ± .24 (5)†	100
	10	1.96 ± .02 (4)	74	1.39 ± .06 (5)	81
	25	1.50 ± .08 (3)	56	1.16 ± .06 (3)	68
Spine	0	4.01 ± .26 (10)	100	3.93 ± .59 (5)	100
	10	4.96 ± .34 (10)	124	5.11 ± .63 (9)	130
	25	4.66 ± .42 (9)	116	4.54 ± .59 (4)	115

* Activity = 10^4 cpm/100 mg/day. † No. of fish analyzed in parentheses.

of incorporation of isotopes by the body of fishes decreases with increasing salt concentration.

The lower rate of incorporation of calcium-45 into the body of lordotic guppies fails to explain the increased calcium content of the animal, and suggests that the turnover rate of body and bone calcium of lordotic guppies is less than that for wild-type animals. In a 25-day experimental period, the rate of turnover of incorporated calcium-45 by the body (Table III) appears to be somewhat less than that for the wild-type guppy but this difference is not significant. In like manner, the turnover rate for spines is the same for both strains of fish. Since the biological half-lives of calcium in guppies have been calculated to be at least 300 and 600 days for body and spine respectively(7), small differences in turnover rate are difficult to detect. The 25-day experimental period is much too short for definitive studies, but the delicate nature of the lordotic guppy prevents us from maintaining these animals under experimental conditions for longer periods of time. Until further information is available, the data presented in this report suggest that the turnover rate of calcium-45 is less for the lordotic guppy than for the normal.

The apparent alteration of calcium metabolism in lordotic guppies and the chemical and biological similarity of calcium and strontium prompted us to study the uptake of strontium-90 from water by the 2 strains of fish. As shown in Table IV, the rate of uptake of strontium-90 from the water in which they swim is the same for both strains of fish under a wide variety of conditions ranging from tap water of low salt content to conditioned water of high salt content. These results are to be expected since the spinal column from lordotic and wild-type fish contain similar quantities of strontium. The data again emphasize the specific alteration of calcium metabolism in lordotic guppies.

Although the rate of uptake of calcium-45 by the body of lordotic fish is lower than the wild-type, the rate of incorporation of calcium-45 by the spinal column is greater for the lordotic than the normal fish, expressed as the ratio of the uptake of the spine to that of the total body (Table II).

The spine:body relationship is significantly different for the two strains of fish when calcium-45 is taken up from tap water of low salt content or from conditioned water of in-

TABLE IV. Uptake and Spine:Body Distribution of Strontium-90 for Wild-Type and Lordotic Male Guppies.

Water*	Body activity ± S.E. Wild-type	Lordotic	Spine:Body ± S.E. Wild-type	Lordotic
Tap H_2O	17.7 ± .9 (7)	15.8 ± .7 (7)	2.44 ± .26 (7)	2.38 ± .24 (8)
Cond. H_2O—{ 75 ppm Ca, 25 ppm Sr }	4.72 ± .11 (4)	5.12 ± .26 (5)	2.12 ± .13 (4)	1.90 ± .22 (3)
Cond. H_2O—{ 200 ppm Ca	2.36 ± .28 (5)	1.97 ± .19 (4)	2.42 ± .20 (12)	2.40 ± .22 (12)
Cond. H_2O—{ 125 ppm Sr	2.16 ± .28 (9)	2.21 ± .22 (9)		

* Water activities ranged between 8.6×10^4 and 3.8×10^5 cpm/ml water.
Mean differences between groups not significantly different.
No. of fish analyzed in parentheses.

termediate salt content. At high concentrations of calcium (275 ppm) this difference is eliminated and the ratio becomes the same.

On the other hand, experiments with strontium-90 indicate that the uptake of this element by the body and the spine:body ratios are similar for both strains of fish. It is interesting to note that the strontium-90 spine:body ratio for both strains of fish is similar to the calcium-45 spine:body ratio of lordotic animals. We have previously shown that the proportion of strontium-90 incorporated into bone, relative to the rate of uptake by the total carcass of wild-type guppies is significantly greater than for calcium-45(9). The significance of these data is obscure at the present time and must await further clarification.

These data demonstrate that the hereditary mutation resulting in lordosis is associated with a specific metabolic abnormality involving calcium metabolism. The mutant guppy, easily raised in large numbers under laboratory conditions, may aid in the elucidation of calcium metabolism and bone formation in vertebrate animals, but a complete understanding of this mutant fish must await further, more definitive studies.

Summary. The lordotic mutation in the guppy is associated with a lower accumulation of body calcium and increased incorporation of calcium into bone. Strontium incorporation is the same for both strains of fishes. These studies indicate a specific defect in calcium metabolism which may further an understanding of calcium metabolism and bone formation.

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Hamster Kidney Cell Tissue Cultures for Propagation of Japanese B Encephalitis Virus.* (23827)

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Since 1949, there has been much developmental laboratory work done with monkey kidney and HeLa cell tissue cultures and the more stable viral agents such as polio, Adeno, and ECHO groups. However, these cell systems frequently have failed to yield marked and consistent cytopathogenic effects (CPE) following inoculation with more labile agents as exemplified by arthropod-borne group. Consequently, the search for new tissue culture systems to permit an *in vitro* methodology for these less stable viruses is continuing. The earlier work has been admirably reviewed by Sanders, Kiem and Lagunoff(1). Syverton and Scherer(2) reported serial propagation of Western (WEE), Eastern (EEE), West Nile (WN), St. Louis (SLE), and Japanese B (JBE) encephalitis viruses in HeLa cell tissue cultures noting irregular and inconsistent CPE with SLE and JBE agents. McCollum and Foley(3) reported successful propagation of JBE virus in monkey kidney, chick fibroblasts, and Detroit-6 cell line tissue cultures, but without complete CPE. Banta(4) reported attempts to cultivate JBE, WEE, WN, and dengue (type

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1) agents in a variety of cell lines and consistent with Scherer's findings, observed CPE of WEE and WN in HeLa cells and in addition, in Henle's human intestinal cells. His infectivity tests revealed evidence of multiplication of all 4 viruses in monkey kidney cells. Bhatt and Work(5) using monkey kidney and chick embryo tissue cultures to propagate WN, Tamil Nad and JBE viruses have obtained better CPE. However, they record inconsistencies of plaque formation and inhibition of CPE. They report loss of mouse pathogenicity following serial passage of JBE virus. Kissling(6) directed efforts primarily towards isolation of virus from tissues of infected animals and arthropods utilizing dog, guinea pig, hamster kidney cell and chick embryo tissue cultures. His results firmly establish the usefulness of hamster kidney cell tissue cultures for detection of EEE, WEE, Venezuelan, SLE, JBE, Murray Valley and Ilheus encephalitis viruses. Our purpose is to report routine preparation of monolayer roller tube tissue cultures from trypsinized hamster kidney by methods essentially identical to those employed for processing of monkey kidney tissues and to extend Kissling's findings regarding usefulness of these cultures as self-sufficient bio-assay system for certain members of arthropod-borne viruses. Evidence is presented that JBE agent has been serially propagated in this cell system and identified after passage by usual serological neutralization test. This demonstrates that *in vitro* method for study of arthropod-borne agents in the laboratory is feasible.

Materials and methods. Preparation of tissue cultures. In the beginning of our studies monolayer tissue cultures from hamster kidneys were prepared by the trypsinization procedures recently summarized by Melnick(7), with the following exceptions: (1) growth medium contained antibiotics to a final concentration of 400 units of penicillin, 2 mg of streptomycin, 20 units of polymyxin, and 25 units of mycostatin/ml; (2) 4% calf serum in 0.5% lactalbumin hydrolysate in Hanks balanced salt solution was used routinely for outgrowth and maintenance of cultures; (3) one pair of kidneys was processed at a time, age or sex was not considered in selection of animal; (4) no attempt was made to remove the pelvis of the hamster kidney; (5) one-half hour of pre-incubation at 37°C in approximately 50 ml of trypsin was employed, and stirring was carried out in approximately 50 ml. Usually 5 successive mixings and decantings at 7-minute intervals were sufficient to provide the desired volume of packed cells for the inoculum. The average yield was approximately .44 ml packed cells/pair of kidneys, which quantity was sufficient to seed 300 ml of medium. When dispensed to tubes in 1 ml amounts, the final number of cells/tube was approximately 150,000. More recently we employed trypsinization of a single pair of kidneys in 100 ml of trypsin for 3 to 4 hours at room temperature with equal success. Outgrowth of cells into a monolayer was generally complete in from 4 to 5 days, then tubes were shaken vigorously, the spent fluid decanted, and new medium with pH of 7.8 to 8.0 added. After overnight incubation the tubes were inoculated. Then tubes were maintained in a stationary slanted position at 37°. Monolayer cultures of hamster cells in glass bottles have also been prepared. However, demonstration of viral plaques has not been successful to date. *Virus preparations.* Aliquots of frozen virus suspensions prepared according to procedures outlined by one of us(8) served as the inoculum for our observations. Mouse and tissue culture passage levels of these viruses are indicated in the charts and tables of the *Results* section. In tests with JBE virus the Nakayama strain was employed. *Diluent.* Ten % normal calf serum (free from JBE antibody) in 0.5% lactalbumin hydrolysate medium served as diluent for titration of virus. *Harvest and storage* of infected tissue culture fluids was accomplished by addition of equal volume of normal calf serum to each tissue culture tube, shell freezing and then thawing at 37°C. The 50% fluids so obtained were pooled, dispensed to ampules in 0.5 ml to 5.0 ml, shell frozen and stored at –70°C. This procedure was deemed necessary to afford adequate protein protection for the labile JBE virus during freezing, thawing and storage. *Assays for viral activity* were accomplished by titrations in either tissue culture tubes or intracerebrally in mice.

TABLE I. CPE* of Selected Viruses on Hamster Kidney Tissue Culture, Dose 0.1 ml.

Viral agent	Group†	Mouse passage	Mouse titer‡	Range of dilutions tested§	End point CPE	Days observed‖
			Marked			
JBE	B	46	7.0	1–5	>5.0	3–5
WN	B	5	7.2	"	4.5	3–4
Uganda S	B	22	6.0	"	4.2	3–6
Ilheus	B	28	7.5	"	>5.0	3
Bunyamwera	?	39	6.6	"	"	3
Semliki Forest	A	8	7.2	"	"	3
			Moderate			
SLE	B	?	6.6	1–5	>5.0	3–5
Dengue I	B	121	?	1–3	1.5	5
" II	B	22	?	1–4	3.5	5
" Trinidad	B	54	?	1–3	2.5	5
			Negative			
Ntaya	B	17	5.9	1–5	<1	7
Zika	B	149	6.8	"	"	7
Bwamba	B	39	4.7	"	"	7
Polio I				1	"	7
" II				1	"	7
" III				1	"	7

* Cytopathogenic effect. † Casals' and Brown's serologic grouping. ‡ Reciprocal of log of mouse LD_{50}. § Reciprocal of log dilution. ‖ Range of days on which CPE became definite from the lowest virus dilution employed to the highest dilution showing CPE; or, if negative, the period of days cultures were observed.

For tissue culture tubes the unit volume of inoculum was 0.2 ml of 50% tissue culture fluid for routine passages or 0.1 ml of appropriate dilutions for titrations; for mouse inoculations the unit volume of inoculum was 0.03 ml. Neutralization tests for JBE virus were performed in usual manner for virus dilution tests and incubation was carried out for 2 hours at 37°C. *Screen tests for cytopathogenic effect* of certain arthropod-borne viruses were performed by thawing the stock mouse brain virus suspension at 37°C, diluting in 10% calf serum diluent and inoculating 0.1 ml of the indicated dilutions into each of 3 to 5 tubes of hamster kidney cell (HKC) tissue cultures. Daily microscopic observations were then made and results recorded as marked, partial or negative, according to whether or not all cells in the culture were markedly altered in appearance, only part of the cells were altered morphologically, or there was no observable difference between inoculated and control tubes maintained under identical conditions. No attempt was made to change the fluids in the cultures. If pH dropped excessively, it was adjusted by addition of a drop of sterile 1.4% $NaHCO_3$ solution.

Results. Susceptibility of hamster kidney tissue cultures to cytopathogenic effects of a variety of viruses. Table I records the findings noted when screen tests for cytopathogenic effects (CPE) of 13 arthropod-borne viruses and 3 polioviruses were performed. The screen tests were done as described above, and in Table I is recorded the range of dilutions used and the endpoint of the cytopathogenic effect. Moreover, the range of days on which CPE first was definite is indicated and also the longest period of observation when no CPE was noted.

It is apparent that arthropod-borne viruses tested fell into 3 groups as regards capacity to produce CPE within observation period of approximately one week. Moreover, this division into a group of those 6 agents which displayed marked CPE within 3 to 6 days, those 4 viruses which revealed only moderate CPE after 4 to 7 days, and those 3 agents which were without effect after 7 days' stationary incubation was not readily related to serological classification of these viruses according to serological groupings of Casals and Brown(9). Further work was limited to observations with Japanese B encephalitis virus as a representative member of Casals' Group

B for which no suitable tissue culture methods were available for routine, practical application.

Cytopathogenic effect of JBE virus. Fig. 1 illustrates the sequential appearance of hamster tissue following inoculation with JBE virus. The captions are self-explanatory and the photomicrographs at 24 and 72 hours post-inoculation reveal that marked morphological changes can frequently be noted as early as 24 hours following inoculation. In contrast to findings reported for the Detroit-6 cell line(3) no evidence of regeneration of tissue in cultures maintained for 21 days follow-

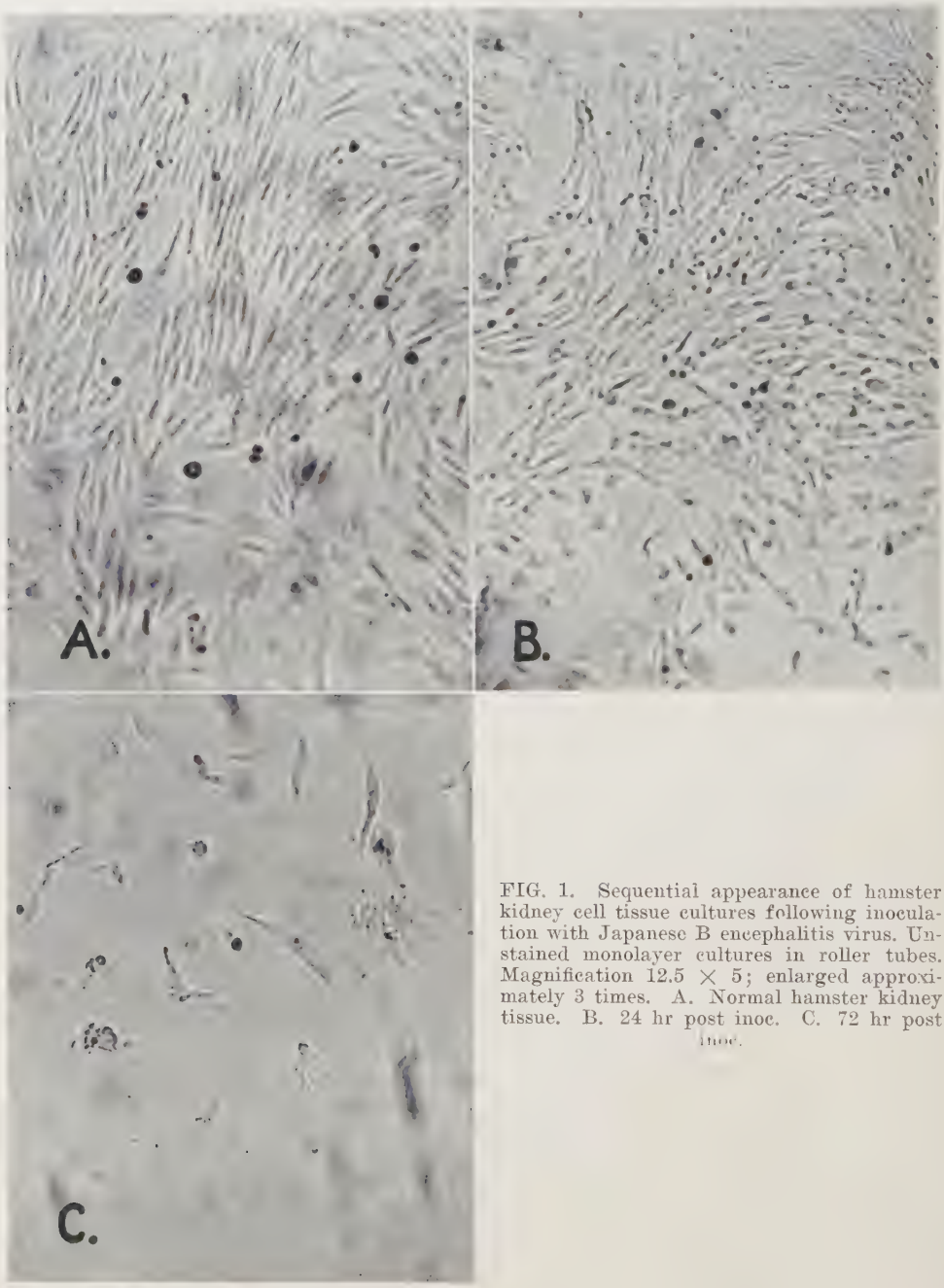

FIG. 1. Sequential appearance of hamster kidney cell tissue cultures following inoculation with Japanese B encephalitis virus. Unstained monolayer cultures in roller tubes. Magnification 12.5 × 5; enlarged approximately 3 times. A. Normal hamster kidney tissue. B. 24 hr post inoc. C. 72 hr post inoc.

TABLE II. Serial Passage of JBE Virus in Hamster Kidney Cell Tissue Cultures.*

Passage level	Dilution of mouse brain (neg log)	Mouse doses in inoculum† (6.9 ×)	Titer of yield‡ doses§/ml (× 1,000,000) Mice	TC	Day of harvest
P-47	3				
TC 1	4	100,000	7.	2	3
2	5	10,000	24.	5	2
3	6	1,000	189.	50	2
4	7	100	93.	10	2
5	8	10	1.9	2	2
6	9	1	1.9	20	2
7	10	.1	6.	20	2
8	11	.01	1.2	10	2

* Japanese B encephalitis virus in 47th mouse passage with LD_{50} titer of $10^{-8.87}$ used for inoculum; 0.1 ml of 1:1,000 dilution used to initiate cultures with subsequent transfers consisting of 0.2 ml of 50% tissue culture fluid in normal calf serum.

† Providing that no loss occurred.

‡ Tissue culture fluid titrated in 0.03 ml volumes in mice and 0.1 ml volumes in tissue cultures.

§ CPE_{50} for tissue cultures; LD_{50} for mice.

ing inoculation has been noted. It has been noted that with certain tissue culture yields the CPE has been delayed or markedly reduced in intensity when 50% tissue culture fluids or 1:10 dilutions of these fluids are inoculated into HKC. This finding, which suggests an interference effect, has not yet been adequately explained. In general, however, with virus dosages greater than 200 TCD_{50} CPE is complete within 72 hours. With smaller doses, 5 to 7 days are required for maximum effect. It should be recorded also that the JBE CPE has been an all-or-none phenomenon in dilutions of virus greater than 1:100. Thus, if even a few cells show evidence of morphological changes, the entire tube is eventually destroyed.

Serial propagation of JBE virus. The initial attempt at serial passage failed after 3 transfers, presumably because high concentrations of serum were not supplied to the tissue culture fluids at time of harvest prior to storage and subsequent passage. The second series of passages was accomplished by adding normal calf serum prior to storage at –70°C. Passages were intentionally not made on day of harvest, since any practical laboratory procedures must not depend on maintaining continuous passage of the test virus.

The results of serial propagation of JBE virus through 8 passages are summarized in Table II. Here serial passage in HKC resulted in 10^{-11} dilution of the original mouse brain, which is well beyond the extinction point of original infectivity. Moreover, tissue culture fluids harvested maintained a fairly constant titer of infectivity for the HKC of approximately 10^{-6}. Therefore, the original mouse brain suspension after a 10^{-11} dilution still contained essentially as many or more infective particles as it did originally, thus indicating that multiplication had occurred. In contrast to HKC titers, mouse infectivity titers have been more irregular and may be indicative that the virus is undergoing an adaptation to tissue culture.

Identification of passage virus by neutralization tests. After 6 serial passages in tissue culture the virus was identified by virus dilution neutralization procedure using as indicator of viral activity both the intracerebral inoculation of mice and the cytopathogenic effect of the virus on hamster kidney tissue cultures. Virus dilutions of 10^{-2} through 10^{-6} were employed with the immune serum using 4 cultures or 6 mice for each dilution. Titrations with normal control serum were carried out in dilutions of 10^{-3} through 10^{-7}. Essentially complete protection was afforded in all dilutions by the immune serum, giving a virus titer of less than 10^{-2} while virus titration in the presence of normal serum gave $10^{-5.5}$ and $10^{-5.4}$ respectively in tissue cultures and in mice. Thus, the results of the 2 tests were essentially identical.

Discussion. We emphasize that information in this report is little more than a progress report and that much additional work must be done before it can be said that there now exists a usable and practical tissue culture method for laboratory study of additional members of the arthropod-borne viruses. However, it is interesting to speculate upon certain important results which could be foreseen if a truly practical "test tube method" were available for routine use. First, it would mean that many of our smaller public health laboratories would have at hand a convenient method for supporting the physician's clinical diagnosis of arthropod-borne viral en-

cephalitis infections by attempted direct isolations of the virus. Secondly, it should be possible to gain fundamental knowledge about the mode of spread of the encephalitis viruses within a monolayer of cells or through the fluid medium such as we now have for poliovirus and others of the more stable viruses. Thirdly, such a system may well provide a source of relatively pure virus particles for preparation of diagnostic antigens or inactivated vaccines. Lastly, continued passage of virus in tissue culture may lead to selection of attenuated strains of virus suitable for production of live virus vaccines such as are now available for prophylaxis against yellow fever.

Summary. (1) Monolayer HKC tissue cultures can be prepared on a routine basis by methods essentially identical with those currently in use for preparation of monkey kidney tissue cultures. In screen tests with 13 arthropod-borne agents and 3 types of poliovirus, HKC proved markedly susceptible to the CPE of Japanese B encephalitis, West Nile, Uganda S, Ilheus, Bunyamwera, and Semliki Forest viruses, only moderately susceptible to effects of the St. Louis encephalitis agent and the New Guinea C, Hawaiian, and Trinidad strains of dengue virus. In contrast, the Ntaya, Zika and Bwamba agents and the 3 types of poliovirus failed to show CPE. (2) Evidence has been presented for serial propagation of JBE virus through 8 serial transfers in HKC tissue cultures. CPE was eventually complete. After 6 passages the virus yield was greater than the starting inoculum, although the dilution factors had carried beyond the extinction point of infectivity of the original inoculum. (3) Identity of the Japanese B encephalitis virus after 6 serial passages in tissue culture was established by neutralization tests performed both in tissue cultures and in mice and thus indicates that the "test tube" procedure may prove useful as a serological tool in the laboratory.

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Reactivity of Globulins from Rheumatoid Sera in the Latex-Fixation Test.* (23828)

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While discussing the various parameters of the latex-fixation test for rheumatoid arthritis, Singer and Plotz(1) reported that 11% of 150 rheumatoid sera agglutinated a standardized suspension of latex particles from which globulin had been deliberately omitted. These sera were those which were strongly positive with the standard "antigen", *i.e.* with a latex particle suspension containing 25 γ of human γ-globulin/ml. The tentative explanation presented was that, in such instances, the latex particles became coated with γ-globulin, or a fraction thereof, from the pa-

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tient's own serum and subsequently were agglutinated with another serum fraction. In the companion paper, these authors(2) found, while comparing the reactivity of rheumatoid sera by the sheep cell agglutination technic and by latex-fixation, that 5 of 107 sera positive by the latter test were reactive only when undiluted. The total number of reactive undiluted sera was not determined.

We reported(3) that the disfunction of normal undiluted sera (human and animal) as the source of "antigen" globulin when mixed with a suspension of latex particles is restored by dilution or by testing the globulins recovered after Na_2SO_4 precipitation. In view of these findings and those of the above authors, it seemed advisable to investigate the possible role of albumin in the demonstration of the agglutinating (or flocculating) factor present in undiluted rheumatoid sera and to relate these results, if possible, to some distortion of serum components.

Materials and methods. Sera were obtained from blood samples of patients attending Rheumatology Clinic of the University Hospital. These sera were maintained in the frozen state and thawed only when aliquots were needed for serologic testing. *Latex-fixation test.* Details of the technic have been described(4). The test is similar to that suggested by Singer and Plotz(1), and differs principally in the quantities of reagents required and length of incubation. *Serum fractionation.* The Na_2SO_4 precipitation procedure(5) is a modification of more standard methods and designed for fractionating small quantities of serum (1 ml). Simplicity of fractionation permits simultaneous treatment of multiple samples. The reconstituted globulins are free of albumin and contamination of the albumin supernate with globulins is minimized. *Paper electrophoresis.* Stained patterns of serum samples subjected to electrophoresis in a Durrum-type apparatus were quantitated by photoelectric scanning with simultaneous area integration (Spinco Analytrol).

Results. Frequency with which undiluted sera react positively in the latex-fixation test. One hundred and seven sera, collected from patients, were diluted 1:20 and tested by the latex-fixation test. Of these, 94 reacted positively and were from cases of typical peripheral rheumatoid arthritis. The remaining 13 sera were from patients ill with other collagen disorders or from patients tentatively suspected of having rheumatoid arthritis. All sera then were tested in the undiluted state, but with the same latex-globulin "antigen". The only undiluted sera found to react positively were among the group which also gave positive tests when diluted 1:20, as in the standard screening procedure(4). Thirty-four % (32 sera) reacted in this manner.

TABLE I. Effect of Precipitating Globulin on Reactivity of 12 Rheumatoid Sera.

Serum No.	Reactivity by latex-fixation test when: Titrated as serum (initial dilution 1:20)	Titrated as globulin (initial dilution 1:20)
1	5120*(—)†	5120 (3+)
2	" (—)	" (4+)
3	2560 (3+)	" (3+)
4	" (—)	2560 (2+)
5	1280 (—)	" (2+)
6	" (—)	1280 (3+)
7	" (—)	640 (2+)
8	640 (—)	1280 (2+)
9	320 (2+)	320 (2+)
10	" (—)	160 (1+)
11	160 (—)	320 (1+)
12	80 (—)	80 (1+)
13–22	— (—)	— (—)

* Titers expressed as reciprocals of dilutions.
† Results in parentheses indicate intensity of reactivity recorded as —, ±, 1+, 2+, 3+, 4+. In first column, results are from tests performed with undiluted sera; in second column, from tests conducted with reconstituted, but undiluted globulins.

Reactivity of globulins recovered after precipitation. A large group of rheumatoid and other sera were diluted 1:20 and screened by the latex-fixation test. Subsequently, the positive sera were titrated and 12 were selected for experimentation on the basis of their varied titers. Ten other sera, negative by the screening test, were included for purposes of control. All sera then were tested in the undiluted state for reactivity (Table I). Only 2 of the undiluted sera were positive, and these, as before, were from rheumatoid patients. Peculiarly, this reactivity was not confined to those sera exhibiting higher titers (to be discussed). One ml of each serum was treated with Na_2SO_4 and the precipitated globulins, reconstituted to original volumes,

TABLE II. Relationship between Latex-Fixation Titer, Activity as Undiluted Sera, and Albumin Content.

Serum No.	Reactivity by latex-fixation of diluted sera (titer)	% albumin
1	5120*(2+)†	56
2	" (—)	78
3	" (2+)	
4	1280 (±)	
5	320 (—)	
6	160 (—)	
7	" (±)	70
8	" (—)	90
9	" (—)	
10	— (—)	87
Normal	— (—)	100

* Titers expressed as reciprocals of serum dilutions.

† Intensity of reactivity recorded as —, ±, 1+, 2+, 3+, 4+, and represents activity of undiluted sera.

were re-examined for reactivity with latex-globulin "antigen". The globulins from all rheumatoid sera (those reactive at dilutions of 1:20 or above) reacted positively, despite the fact that only 2 such sera were reactive when undiluted (Table I). The globulins from sera which were negative when diluted to 1:20 uniformly failed to demonstrate reactivity. Titrations of the flocculating component of the globulins were performed in the usual manner with latex-"antigen". As seen from the Table, virtually no alteration in reactivity resulted from the precipitation procedure.

Since, seemingly, removal of albumin (Table I) conferred reactivity to reconstituted, but undiluted, globulins, it seemed possible that reactivity of undiluted sera might be a reflection of the distribution of serum components. Accordingly, 9 rheumatoid sera, varying in latex-fixation titers and in reactivity when undiluted, were subjected to paper electrophoretic analysis. A 10th serum from another collagen disease, and non-reactive by the latex-fixation test, was included as was a normal serum. The activities of these sera are recorded in Table II.

For purpose of illustration, the albumin content of sera 1, 2, 7, 8 and 10 are included in the Table. The normal serum was subjected to electrophoresis simultaneously with the test sera to facilitate accurate comparisons. The albumin content of normal serum was represented as 100%. Concentrations of albumin in the test sera were calculated to this reference. It should be recalled that the normal serum was from a single blood specimen and while concentrations of the component protein fractions were within the normal range, they do not correspond necessarily to the averaged concentrations of normal serum proteins.

A comparison of the albumin content of test serum 1 and of normal serum readily reveals the decreased albumin content of test serum 1 (56% of normal). In view of the high concentration of latex-flocculating factor in this serum, it is not surprising that this serum reacted positively when undiluted. Test serum 2, although demonstrating the same latex titer as serum 1 when diluted, contained significantly more albumin (78%). This serum was non-reactive in the undiluted state. Sera 7 and 8 exhibited the same modest latex-fixation titer of 1:160. However, serum 7 reacted as undiluted serum with the latex-globulin "antigen". As before, the albumin content of this serum is decreased (70%) while the concentration of albumin in serum 8 more nearly approaches that of the control serum (90%). The albumin content of the non-reactive serum 10 (87%) also is similar to that of normal serum.

The concentration of albumin in the remaining 5 sera bore the same relationship to reactivity in the undiluted state as did the sera just described.

Discussion. In experiments not reported here, attempts to definitely assign the inhibitory property of whole serum to the albumin fraction met with failure. Reconstituted sero-reactive globulins were mixed with appropriate quantities of human albumin and subjected to the latex-fixation test. In no instance was inhibition observed. These findings, however, do not eliminate albumin from consideration, inasmuch as it is well established that mere admixture of serum components does not endow the mixture with the properties of whole serum.

It should be understood clearly that the inhibition described here and correlated with serum albumin is distinct from the inhibitor reported by Heller *et al.*(6) who were re-

ferring to the inhibitor of agglutination of sensitized sheep cells by rheumatoid sera and assigned this activity to some component of the FII fraction of serum. Based on this observation, tanned sheep erythrocytes were coated with γ-globulin and employed for detection of the agglutinating factor in rheumatoid sera. The test for agglutination-inhibitor as devised by Ziff *et al.*(7) is associated with the euglobulin fraction of serum. It was postulated that all sera probably contain inhibitor, but that it cannot be readily demonstrated in the euglobulin fraction of rheumatoid sera because of neutralization by the rheumatoid factor. Albumin was found to be free of inhibitive properties by both groups of workers.

In the present report the influence of serum albumin in the latex-fixation technic most probably is a function of its protective colloid properties and may reflect certain, as yet unexplained, aspects of the test.

Summary. 1. Thirty-two of 94 rheumatoid sera, positive by the latex-fixation test when diluted 1:20, also reacted in the undiluted state. 2. Sodium sulfate precipitation of the remaining 62 sera permitted positive demonstrations with the reconstituted globulins. 3. No alteration in titer was associated with the precipitation procedure. 4 Electrophoretic patterns revealed that the reactive undiluted sera manifested a reduced albumin content.

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Biological Activity of Soluble Antigen-Antibody Complexes I. Skin Reactive Properties.* (23829)

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As a result of some preliminary studies made in conjunction with an investigation on the effect of soluble complexes on smooth muscle(1), it became obvious that soluble antigen-antibody complexes formed in excess antigen also produced a marked increase in permeability of capillaries in guinea pig skin. This was shown by the fact that when a small amount of soluble antigen-antibody complex was injected into the skin of normal guinea pigs which had previously received an intravenous injection of Evans Blue, the area of injection became blue. The following paper presents a more detailed investigation of this phenomenon. The first work involved fractionation of antiserums by starch electrophoresis to determine if the antibody involved was a precipitating gamma globulin. The second study was made on normal and immune serums without the addition of any antigen when it became apparent that immune as well as normal serums contained a skin-irritating protein component.

Materials and methods. Antiserums: In the present investigation, only Armour's bovine serum albumin (BSA) and rabbit antiserum were studied. Three different pools (1, 2 and 3) of antiserum were used but immunization was the same in all rabbits. The schedule consisted of 10 mg of BSA intravenously 3 times a week for 4 weeks, then bleeding 7 days after the last injection. Serum from each rabbit was titrated by qualitative

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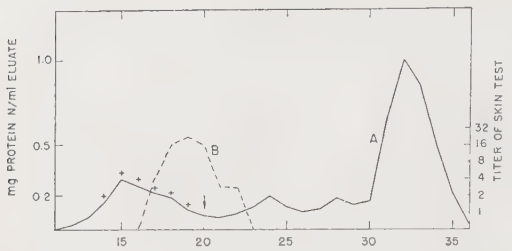

FIG. 1. Starch electrophoretic pattern of rabbit anti-BSA No. 1. Curve A represents conc. of protein N in eluates. Curve B indicates titer of skin test. Plus indicates presence of precipitating antibody. Arrow indicates the origin.

tests for content of precipitating antibody and those having approximately the same titer were pooled and the pools stored at –40°C. The amount of precipitable antibody N per ml in the 3 different pools was, No. 1 = 650 μg, No. 2 = 145 μg, and No. 3 = 369 μg. *Zonal electrophoresis* on starch blocks was carried out in the usual manner using barbital buffer pH 8.6, μ = 0.10. Serum samples of about 6 ml were allowed to separate for about 19 hours at 60 MA. The starch block was then cut into 1.0 cm segments and each segment placed in a 50 ml centrifuge tube and eluted with 9 ml of saline. The amount of protein in each eluate was determined by biuret analysis(2). *Skin reactions* were all made on guinea pigs by a modification of the method described by Ovary(3). Guinea pigs weighing about 500 g were given intravenously 1.5 ml of a 0.5% solution of Evans Blue per kg body weight. Test solutions consisting of 0.1 ml were then immediately injected intracutaneously in the shaved lumbar back surface of the "blued" animals. Control sites consisted of 0.1 ml of pyrogen-free saline and 0.1 ml pyrogen-free saline containing 0.3 μg of histamine phosphate. A positive reaction became quite apparent in 2-4 minutes and reached maximum in 20-30 minutes. Animals were sacrificed 30 minutes after injection and the test area of the skin removed. The reactions were graded by appearance and average diameter of blueing according to the following scheme: negative, less than 5 mm; 1 plus, 5 to 7 mm; 2 plus, 7 to 10 mm; 3 plus, more than 10 mm and deep color.

Results. A. *Soluble antigen-antibody complexes.* The first studies were made on whole serums No. 1 and 2. Skin tests were made on the supernatants obtained from precipitin reactions. The precipitin reactions were set up in the usual manner in which equal volumes of various 2-fold dilutions of antigen and undiluted antiserum were mixed, allowed to stand for 48 hours at 4°C and the supernatants obtained by centrifugation. Each supernatant was then tested in various dilutions (from undiluted to 1:128). Supernatants from tests made with serum No. 2 showed maximum skin reaction in slight antigen excess (1:64) and less at equivalence and antibody excess (1:16 to 1:32). With serum No. 1 there was a slight tendency for skin reactions to be strongest in supernates from the equivalence region and slight antigen excess, but fairly severe skin reactions were obtained in dilutions of 1:64 over the entire range of antigen-antiserum tested. It became apparent upon testing the antigen and the antiserums separately that the antiserum alone contained an irritating component.

In view of this finding, the following experiment was carried out to determine whether the irritating substance could be separated from precipitating antibody by electrophoretic fractionation on starch. Six ml samples of antiserums No. 1 and 2 were allowed to migrate on a starch block for 19 hours, then cut into 1.0 cm segments and the protein eluted from each segment. Each eluate was tested for precipitins by ring test and for skin activity. The results are given in Fig. 1 and 2. Some over-lapping occurred in the region of "fast gamma" but a few eluates in "slow

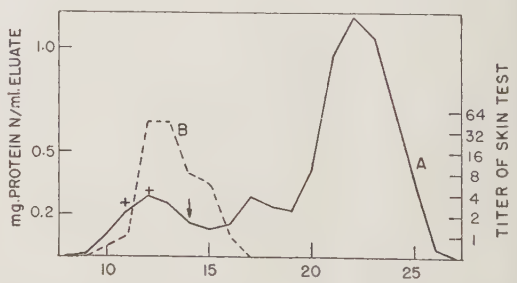

FIG. 2. Starch electrophoretic pattern of rabbit anti-BSA No. 2. Curve A represents conc. of protein N in eluates. Curve B indicates titer of skin test. Plus indicates presence of precipitating antibody. Arrow indicates the origin.

TABLE I. Activity of Supernates from Quantitative Precipitin Analyses.

Antigen N added	Total N precipitated	Antibody N precipitated	Tests on supernate	
	μg		Presence of Ag or Ab	Skin test
1 ml, slow γ globulin fraction from antiserum No. 2 .25 ml of antigen were used				
80	0	0	Excess antigen	+++†
40	7.6		*Idem*	+++
20	41.7		"	+++
10	63.4	ca 53.4	Trace antigen	+++
5	51.9	46.9	Neither	—
2.5	30.3	27.8	Trace antibody	—
1.25	14.8	13.5	Excess "	—
0	0	0	Antibody	—
2 ml, slow γ globulin fraction from antiserum No. 3 .5 ml of antigen were used				
160	1.9		Excess antigen	1:4*
80	9.5		*Idem*	1:8
40	52.6		"	1:4
20	89.6	ca 69.6	Trace antigen	1
10	87.4	77.4	Neither	—
5	52.5	47.5	"	—
2.5	26.8	24.3	Trace antibody	—
0	0	0	Antibody	—

* Minimum conc. of supernate required to produce skin reaction.

† Similar reactions were obtained by 1:2 dilution.

BSA (320 μg N/ml) produced no reaction.

gamma" of serum No. 1 gave only precipitin activity and a few in the area between gamma and beta gave only skin activity. The low titer serum No. 2 showed over-lapping in the slow gamma area, but skin activity and no precipitin in the fast gamma. The three eluates (No. 14, 15 and 16) of serum No. 1 were then combined and antigen added in varying dilutions. The results of this test are given in Table I. It became apparent from these data that the precipitating antibody would produce an irritating complex with antigen in the region of antigen excess and none in antibody excess.

A similar study was made on a gamma globulin fraction from antiserum No. 3. The gamma globulin was first isolated by repeated precipitation with ⅓ saturated ammonium sulfate solution at pH 7.8. After dialysis a 4.0% solution was fractionated by starch block electrophoresis and eluates from 1.0 cm segments tested for precipitins and skin activity. The results were essentially the same as obtained previously for the whole antiserum. Again the slow moving gamma components which contained precipitin, but no skin reactive substance, were combined and aliquots mixed with varying amounts of antigen. The results are shown in Table I.

Some idea of the quantitative aspects of the activity of the soluble antigen-antibody complex was obtained from the following experiment. An antigen-antibody precipitate was formed by mixing a sample of the slow gamma obtained from antiserum No. 3 with an amount of BSA indicated in Table I at the equivalence point. The precipitate was washed 3 times with cold saline and then suspended in 2 ml of saline containing 128 μg N of BSA. The mixture was shaken for 18 hours at 4°C, the small amount of residue removed, and the solution tested for skin activity. Since the amount of antibody N in the original precipitate was approximately 78 μg N, the final solution contained less than 39 μg/ml. This solution gave a good positive skin reaction in dilutions of 1:16 and of 1:32. In order to obtain similar skin reactions with histamine phosphate, an amount of about 1.2 μg/ml was required. Assuming that the skin-reactive material from antigen-antibody mixtures was a soluble complex, then the limiting molecular weight would be of the order of 300,000 [$Ag_2Ab(4)$] and on a molecular basis would be more than 100 times as active as histamine.

B. *Activity of normal and immune serums without addition of antigen.* As was mentioned previously, serums without the addition of any antigen usually produced some local diffusion of dye into the skin of "blued" animals. In general, serums from normal rabbits gave a definite skin reaction in dilutions less than 1:10 while immune serums usually produced similar reactions in dilutions of 1:32 to 1:64. This effect of normal serum alone has been the subject of much investigation [*e.g.* Wilhelm *et al.*(5) who found an active component in alpha globulin]. However, in the present investigation the occurrence of the skin-reactive material in the gamma component of serum and the marked increase in activity following immunization suggests the possibility of some sort of antigen antibody mechanism.

The skin-irritating material of antiserum occurred in what is commonly considered fast gamma and slow beta (Fig. 1 and 2). It also occurred in the same general region of normal serum. This electrophoretic characteristic is essentially the same as found by Aladjem *et al.*(6) who obtained immediate-type skin reactions in normal human volunteers with fractions from immune rabbit serums. The skin reaction produced in the present study in blued guinea pigs was essentially the same as that produced by soluble antigen-antibody complexes, histamine or by the injection of antigen into the skin of a sensitized guinea pig. A positive reaction became quite definite in 2-3 minutes and reached a maximum in 30 minutes. The investigation of the nature of the skin-irritating material in immune and normal serum is not complete. However, preliminary studies indicate that it is a nondialyzable, heat-labile protein component that can be concentrated by centrifugation at 100,000 g as described for isolation of Rh agglutinating antibody by Campbell *et al.*(7).

Discussion. There seems to be no doubt that soluble antigen-antibody complexes found in excess antigen are toxic to guinea pigs and induce reactions which are similar to specific hypersensitivity. An explanation of the mechanism involved must await further study but the possible courses for investigation are obvious. In the case of soluble complexes the possibility exists that free antigen or antibody rapidly sensitizes tissue and the reaction observed is the result of a subsequent reaction with the complementary reagent (*e.g.* passive or reverse passive anaphylaxis). Another possibility is that the complex itself is toxic due to molecular changes brought about in either the antigen molecule or the antibody molecule as a result of combination. This leads to a study of many different antigen-antibody systems, including haptens, and to include soluble complexes formed in antibody excess. The third possibility is that the component of complement in antiserum is activated by the soluble antigen-antibody complex and becomes toxic; however, this was excluded by using decomplemented antiserum. Details of the experiment will be described later.

The skin-irritating fraction in normal and immune serum may be a circulating antigen-antibody complex. That of normal serum would not be known and would probably be a mixture of soluble complexes of a large number of antigens to which the animal is constantly exposed in his normal environment.

Summary. Immediate skin reactions were obtained in normal guinea pigs by intradermal injections of soluble antigen-antibody complexes. Immediate skin reactions were also obtained by injections of an electrophoretically separated component of serum from immunized or normal rabbits.

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Chemotherapeutic Activity of Some Sulfapyridine-1-Oxides. (23830)

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Sulfapyridine-l-oxide, and several alkyl substituted derivatives prepared in our laboratories(1), were found to be more acidic than the corresponding sulfapyridines. It was assumed that these compounds might not induce urolithiasis and for that reason their potential as chemotherapeutic agents was investigated in laboratory animals.

Methods. Chemotherapeutic activity of the drugs was determined(2) in *E. coli*-infected mice as follows. Eighteen-hour cultures of *E. coli* (A.T.C.C. No. 26) in dextrose peptone broth were diluted 1 x 10^4 fold, and 1 ml of the diluted culture was mixed with 9 ml of 5% gastric mucin. The dose used for intraperitoneal infection, 0.5 ml of the 1 x 10^5 dilution, contained an average of 6000 cells or 5 to 10 thousand lethal doses. Virulence of the culture was maintained by frequent mouse passage. Test compounds and reference drugs were suspended in 10% gum acacia, and 0.5 ml of the suspension was given by stomach tube. The first dose was given 30 to 45 minutes before infection; the second was given about 6 hours later. The drugs were then given at a rate of 2 doses per day for 2 days, and in some cases, as noted, for 4 days. Control mice were treated in a parallel manner with acacia solution. Groups of 10 or 15 male albino Carworth Farms CF_1 mice were used at each dose level. Some experiments were run in duplicate. In these instances, the data were combined for presentation in Table I. All animals were observed daily for 10 days. To conserve space survivals are recorded in Table I only for the first, second and tenth days. The pKa's of the sulfapyridine-l-oxides and reference drugs as well as their acetylated derivatives were approximated by titrating 0.1% solutions of the drugs in water at 25°C with one-half an equivalent of 0.1 N sodium hydroxide. The data are recorded in Tables I and II for comparative purposes. Using procedure first described by Antopol(3) in his studies of sulfonamide urolithiasis, precipitation within the kidney was sought following intravenous injection of sodium salts of 6 acetylsulfonamides. Groups of 6 Sprague Dawley rats were used at each dose level. Solutions of the sodium salts were prepared in concentrations of 5 and 10% by the addition of approximately 1 equivalent of dilute sodium hydroxide. The pH's were 8 $\pm$ 0.5 except in the case of acetylsulfapyridine where pH was 10.5. Solutions were injected into tail vein at 1 ml/30 seconds. Twenty-four, 48 and 96 hours after administration of the drug, kidneys were removed, sectioned, and examined under dissecting microscope. Urolithiasis, if it occurred at all, appeared within 24 hours. Consequently the data summarized in Table II include only observations recorded after 24 hours. Hypoglycemic activity was assayed according to procedure of Mirsky *et al.*(4). The drugs were dissolved to the extent of 500 mg % in 0.5% sodium bicarbonate solution. Sprague Dawley rats which had been fasted overnight were given either 2 or 5 ml of solution by stomach tube/100 g body weight. Thus, each rat received either 100 or 250 mg of the individual drugs/kg body weight. Groups of 3 to 6 rats were used for each drug. Samples of tail blood were taken shortly before administration of drugs and at 1, 2 and 4 hours thereafter. Blood glucose determinations were performed according to Nelson(5). Averaged data are recorded in Table III.

Results. As shown in Table I, the 6′-methyl derivative is the most active of the sulfapyridine-l-oxides. Shifting the methyl group to any other position in the pyridine ring results in a considerable loss of chemotherapeutic activity. Similarly, increasing the chain length from methyl to ethyl results in a diminution of activity. Under the conditions of these experiments, sulfamethoxypyridazine is clearly the most active of the reference drugs. Sulfamethylthiadiazole and sulfadimethoxytriazine are relatively weak and sulfisoxazole is of intermediate activity. Calculated according to the method of Litchfield

and Wilcoxon(6) the ED_{50}'s for 6′-methylsulfapyridine-l-oxide and sulfisoxazole are 0.54 and 0.65 mg/mouse, respectively. 6′-Methylsulfapyridine-l-oxide appears to be more potent than sulfisoxazole, but the difference is not significant at the 5% level.

In an attempt to decide qualitatively which drugs would and which would not form uroliths, solutions of the sodium salts of various acetylated sulfonamides were rapidly injected intravenously in the rat. In this way, absorption, and detoxication are largely by-passed and a maximum blood concentration of the drug is available for distribution and renal excretion. If the drugs are cleared in the kidney by simple glomerular filtration, tubular reabsorption of water will cause the drugs to precipitate only if their solubilities in the tubular urine are exceeded. The solubilities of these acetylsulfonamides are determined primarily by the extent to which they undergo salt formation at the pH of the plasma filtrate. As shown in Table II, acetylsulfisoxazole, acetylsulfamethylthiadiazole, and 6′-methylsulfapyridine-l-oxide, which are 50% ionized at pH levels of 4.4, 5.2 and 5.4

TABLE I. Activity of Sulfapyridine-l-oxides and Reference Drugs against *E. coli* Infections in Mice.

Compound	pKa	No. of mice	Dose (mg/mouse)	Survivals in days (%) 1	2	10
Sulfapyridine-l-oxide	5.2	10*	10.	100	100	100
		10*	5.	100	40	20
		10	2.5	50	0	
3′-Methylsulfapyridine-l-oxide	6.1	10	5.0	10	0	
		10	1.0	0		
		10	.5	0		
4′-Methylsulfapyridine-l-oxide	5.5	15	5.0	93	80	80
		15	1.25	33	0	
5′-Methylsulfapyridine-l-oxide	5.7	10	2.5	90	0	
6′-Methylsulfapyridine-l-oxide	5.9	30	1.25	100	100	100
		30	1.0	100	93	93
		40	.5	98	60	45
		10	.4	90	30	20
		10	.3	100	10	0
6′-Ethylsulfapyridine-l-oxide	6.1	10	2.5	70	60	50
		10	1.0	90	80	70
		10	.5	40	10	0
4′, 6′-Dimethylsulfapyridine-l-oxide	6.2	15	5.0	100	87	87
		15	1.25	33	0	
Sulfadimethoxytriazine	5.0	15	2.5	7	0	
		15	1.0	0		
		15	.5	0		
Sulfamethylthiadiazole	5.4	10	1.25	30	0	
		10	.9	30	10	10
		20	.6	25	10	10
		10	.4	30	0	
Sulfisoxazole	5.0	35	1.25	100	94	94
		20	1.0	100	80	80
		35	.6	100	37	34
		10	.4	90	20	20
		10	.3	80	0	0
Sulfamethoxypyridazine	7.2	15	2.5	100	100	100
		15	1.0	100	100	100
		15	.5	100	100	100
Controls 10^{-5}		80		6	1	1
10^{-6}		80		23	9	9
10^{-7}		80		31	8	6
10^{-8}		85		54	14	14
10^{-9}		85		85	56	56

* Animals were treated for 4 days. In all other instances treatment was limited to 2 days.

TABLE II. Urolithiasis following Intravenous Acetylated Sulfonamides in the Rat.

Drug*	pKa	Dose† (mg/kg)	No. dead	No. of animals showing uroliths
Sulfapyridine	8.2	400	1	6
		200	0	6
		100	0	3
Sulfamethoxypyridazine	6.9	1000	1	0
		400	0	0
Sulfadiazine	6.1	400	0	6
		200	0	6
		100	0	2
6′-Methylsulfapyridine-l-oxide	5.4	500	1	0
		400	0	0
Sulfamethylthiadiazole	5.2	1000	0	0
		400	0	0
Sulfisoxazole	4.4	1000	0	0
		400	0	0

* All were acetylated.
† 6 rats were used at each dose level.

respectively, did not precipitate within the tubules. In contrast, acetylsulfapyridine, which undergoes little dissociation in acidic urine, produced uroliths in a high proportion of the animals at doses of 100 mg/kg and above. Acetylsulfadiazine (pKa = 6.1) produced a high incidence of kidney stones; acetylsulfamethoxypyridazine, though a weaker acid (pKa = 6.9), did not. Perhaps in this particular instance, the slow rate of renal excretion of the pyridazine, as noted by others(7), is determinant. Although all the implications of this intravenous procedure have admittedly not been developed, it is an interesting approach to the sulfonamide-urolith problem, and it appears to afford useful information.

TABLE III. Blood Sugar Levels in Rats following Sulfapyridine-l-oxides or Sulfonylureas.

Substituent or drug	Dose (mg/kg)	Mean blood sugar given in % of initial conc. 1 hr	2 hr	4 hr
4′-Methyl	250	85	96	85
5′ ″	250	73	93	104
6′ ″	100	114	104	
6′ ″	250	105	74	80
4′,6′-Dimethyl	250	93	99	99
Tolbutamide	250	73	47	39
Carbutamide	100	79	63	

In the course of evaluation of the sulfapyridine-l-oxides their potential hypoglycemic activity was assayed in the rat. As shown in Table III none produced a significant hypoglycemia.

Summary. 6′-Methylsulfapyridine-l-oxide was found to be a potent chemotherapeutic agent in the treatment of *E. coli* infections in mice. It did not cause hypoglycemia in the rat. Intravenous administration of the sodium salt of its acetylated derivative did not lead to urolithiasis.

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Prothrombin Activation Cycle.* (23831)

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It was remarkable to observe that prothrombin loses its activity when mixed with a small amount of thrombin(1). This loss in activity consists of becoming refractory to activation in the presence of lung thromboplastin, calcium ions and Ac-globulin (2-stage analytical reagents). When the proportion of thrombin to prothrombin is increased the prothrombin not only loses its activity but also partly regains it again, and then finally becomes thrombin. All this takes about 2 days (2). Apparently the same events occur in 25% sodium citrate solution, but far more rapidly(3). The sequence may be described by the following: Prothrombin (sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin-derivative I (not sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin-derivative II (sensitive to calcium + Ac-globulin + lung thromboplastin) → Thrombin and possibly other reaction products.

The prothrombin activation cycle described above is completed when thrombin is added to prothrombin. Perhaps the cycle is also negotiated when prothrombin activates rapidly in the presence of procoagulants, but then this transpires so fast that no means devised thus far is suitable for following the changes. Any phenomena that are natural in accelerated autocatalytic activation of prothrombin might be expected to occur in the molecule left by itself, if no side reactions interfere. For this reason we supposed that prothrombin alone might lose its activity and subsequently regain it. In this brief presentation we describe conditions that fulfill this requirement.

Methods. Prothrombin. This was prepared from bovine plasma by methods developed in this laboratory(4). The last traces of Ac-globulin were not removed. The specific activity was usually near 25,000 U/mg tyrosine. *Prothrombin and thrombin activity.* These assays were performed as previously described(5,6).

Results. A spontaneous activation cycle was first noticed when a lyophilized product of prothrombin was dissolved in physiological saline, buffered with imidazole at pH 7.35 to give a solution containing 11,600 U/ml. This was kept in a stoppered test tube, refrigerated (4°C), and the activity of prothrombin was measured every 2 to 3 days. The prothrombin activity decreased to 5000 U/ml in 4 days but gradually returned to the original level and higher in 15 days. By 29 days it declined to 1000 U/ml and further analyses were impossible because the prothrombin solution was exhausted.

Further studies were then started. A prothrombin product was dissolved in M/15 phosphate buffer at pH 6.0 and 7.2 and a third sample was dissolved in veronal buffer of 0.1 ionic strength at pH 8.6. Each of these solutions contained 5150 U/ml of prothrombin. These were stored at 4°C and prothrombin activity was measured every 2-3 days. The prothrombin activation cycle (Fig. 1) shows itself at each of these hydrogen ion concentrations. The loss and gain in prothrombin activity was without detectable thrombin in the solutions. In experiments previously described(1,2,3) thrombin was added and presumably accelerates the alterations that prothrombin alone can undergo. In these experiments the appearance of thrombin was seen late. After 28 days there were about 100 U/ml of thrombin in the solution of prothrombin at pH 8.6 and thrombin activity increased to 300 U/ml 2 days later. Only a very small amount of thrombin was measured in solutions at pH 6.0 and 7.2 by this time, but a month later there were 140

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† Work completed during tenure of Award in Support of Creative Work from Commonwealth Fund of N. Y.

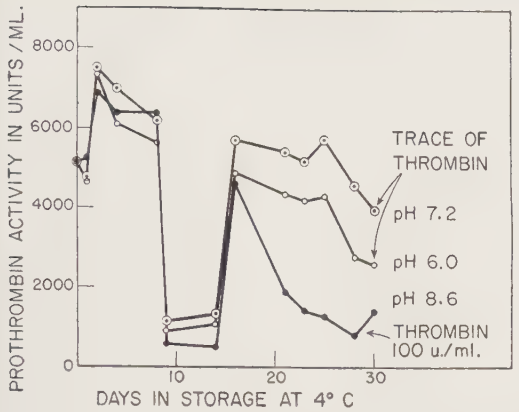

FIG. 1. Changes in reactivity of purified prothrombin stored at 4°C, in M/15 phosphate buffer or veronal buffer 0.1 ionic strength and pH 8.5. Assay by 2-stage analysis.

U/ml and 1050 U/ml of thrombin respectively.

All prothrombin preparations so far used in these experiments were dried from the frozen state. To see whether this process of drying had anything to do with the activation cycle the lyophilizing process was omitted and a prothrombin product in water solution was stored and observed. Two products of prothrombin were followed through the first phases of the cycle illustrated by means of Fig. 1. The original activity of each of these 2 prothrombin solutions was 15,000 U/ml. After 14 or 20 days of storage the prothrombin activity went up to 18,000 U/ml then gradually came down to 10,000 U/ml and finally reached 5000 U/ml in one and 2000 U/ml in the other case. At this point which was 49 days after storage at 4°C thrombin developed in both solutions.

In further experiments prothrombin was studied with respect to salt. Prothrombin (not lyophilized) was dissolved respectively in distilled water, 0.9% NaCl solution and saturated NaCl solution all at pH 7.0. Again the events observed were essentially like that seen in other experiments, however, the prothrombin in the saturated NaCl solution lost its activity more rapidly and did not regain reactivity very extensively. By about 35 days all produced thrombin ranging from 400 U/ml (water and 0.9 NaCl) to less than 1 U/ml (saturated NaCl). Similar results were obtained with a prothrombin product which was first dried from the frozen state.

Discussion. Prothrombin preparations can go through an activation cycle spontaneously, and without detectable thrombin until the last stages. This is most likely a property of the prothrombin molecule itself and does not require the addition of procoagulants or even the activator which is thrombin. Since this is natural to prothrombin, and occurs more rapidly in the presence of thrombin we think this very likely is a cycle that is partly or fully completed with prothrombin activation in our ordinary physiology. Material that has only gone through the inactivation phase could be a constituent of normal plasma. Since no one has observed these changes in rapid prothrombin activation the view is based on inferences based on various observations about slow activation. Moreover, small quantities of brain thromboplastin which is all lipid, or platelet factor 3, which is a lipoprotein, can be mixed with prothrombin and "inactivation" occurs. This is really the beginning of the activation cycle which is not completed because other substances are needed under the circumstances. When such a cycle is started with platelet factor 3 the prothrombin activity can be regenerated with mitochondria(7). So the change is not "irreversible" after activation with platelet factor 3. Whether the regenerated prothrombin activity is the same as the original prothrombin is not known, but we are inclined to favor the view that this part of the cycle is a continuous run-off process involving intramolecular space relationships. These might create configurations refractory to the environment created by the procoagulants and then with further unfolding and/or folding of prothrombin the procoagulants would again favor thrombin formation—the latter actually being the activator.

Summary. The prothrombin activation cycle consists of a sequence described by the following: Prothrombin (sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin derivative I (not sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin derivative II (sensitive to calcium + Ac-globulin + lung thrombo-

plastin) → Thrombin. Solutions of prothrombin standing at 4°C go through most of this cycle, the prothrombin molecule alone possessing the peculiar properties that enables it to do this.

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Plasma Ketosteroid in Adrenal-Tumor Bearing Mice.*†‡ (23832)

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Several investigators have shown that transplantable adrenal tumors in mice may produce steroid hormone. This has been demonstrated by anatomical changes in the hosts, such as the development of atrophic adrenal glands, mammary and uterine changes, or chemical studies either of urinary steroid or *in vitro* response of tumors to ACTH(1-5).

This report concerns the measurement of plasma ketosteroid in CDF_1 hybrid and Balb-c mice bearing a hormonally active, transplantable, adrenal tumor which arose spontaneously in a Balb-c mouse in the laboratory of Dr. H. B. Andervont of the National Cancer Institue.

Materials and methods. Determination of plasma 17-ketosteroids. Plasma ketosteroids were determined by a modification of the procedure of Gardner(6). Each determination represented a pool of plasma obtained from 4 mice. Mice were lightly anesthetized with ether and blood from the severed femoral vein was aspirated into a heparinized syringe. *Tumor inoculation and animal maintenance.* Two months old CDF_1 were injected subcutaneously with 0.1 ml of a 50% tumor-saline emulsion. Tumor-bearing animals were sacrificed on twenty-fifth day after inoculation for plasma steroid determination; control mice which had been maintained under similar conditions were sacrificed simultaneously. Mice were maintained at 24°C and fed Purina Chow.

Results. Tumor growth. After about 5 weeks of subcutaneous growth, the tumor was hemorrhagic and the animal died. Metastases were never observed in unoperated animals. The hosts developed atrophic adrenals; enlarged uteri were observed in females. Gross and microscopic details of tumor growth are being studied by Dr. T. B. Dunn of Nat. Cancer Inst.

The effect of tumor growth in male mice is illustrated in Fig. 1. After 25 days of tumor growth, the ketosteroid level is elevated with respect to the plasma of control mice. Gonadectomy-adrenalectomy decreased the plasma ketosteroid level of control mice to trace values but did not affect the elevated level found in the plasma of tumor-bearing animals. The tumor effected an increase in plasma ketosteroid roughly similar to that caused by administration of 1 mg of ACTH for 5 successive days. An unexpected finding was the presence of widespread gross metastases in such organs as lung, liver, and spleen in 11 of

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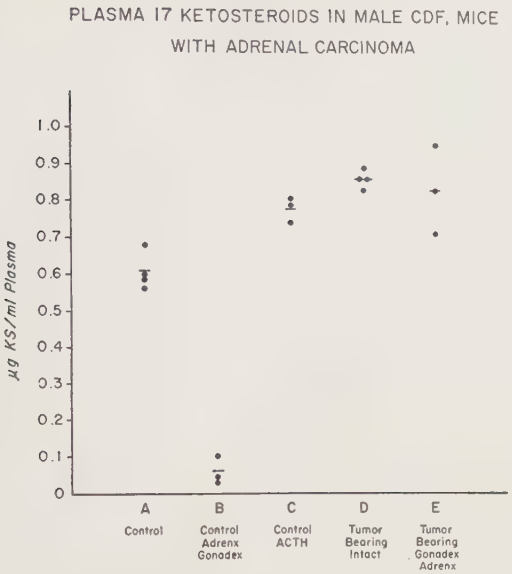

FIG. 1. Adrenalectomy - gonadectomy was performed on a control and tumor-bearing group 20 days after tumor inoculation in the latter group. ACTH was administered subcut., 1 mg daily, to a control group of mice for the last 5 days before sacrifice. Control and tumor-bearing animals were sacrificed after 25 days of tumor growth in the latter group. Each point represents a plasma ketosteroid determination on a pool from 4 mice.

12 adrenalectomized-gonadectomized mice. The development of metastases in these animals suggests that the tumor is not autonomous. This suggests that the tumor is hormonally controlled in unoperated tumor-bearing animals.

The effect of tumor growth on plasma ketosteroid in female mice is illustrated in Fig. 2. After 25 days of tumor growth, the plasma ketosteroid concentration is elevated with respect to the controls. Adrenalectomy decreased the plasma ketosteroid of control mice to trace values but did not affect the elevated level found in the plasma of tumor-bearing animals. Administration of ACTH to control mice caused an increase in plasma ketosteroids but no significant change in plasma concentration in tumor-bearing animals. Administration of Δ1-9α fluorohydrocortisone (Δ1-9FHC) to control mice decreased the plasma ketosteroid level. This powerful ACTH suppressant failed to affect the elevated plasma ketosteroid levels in tumor-bearing mice.

Distant metastases were present in 6/16 adrenalectomized mice. No metastases were found in any other group. Sham adrenalectomy was performed on 20 female tumor-bearing mice and plasma ketosteroid was within the range of unoperated tumor-bearing mice and no metastases were evident.

Discussion. Data have been presented to indicate that in the absence of the testis and adrenal, plasma ketosteroid concentrations fall to trace values. Adrenalectomized female mice bearing the transplanted adrenal tumor have elevated plasma ketosteroid concentrations and adrenalectomized gonadectomized male mice with tumor have elevated plasma levels. These data indicate that the tumor produces the excessive amounts of steroid. Steroid production is independent of ACTH since administration of ACTH, or Δ1-9α FHC produces no change in the plasma level.

Gallagher has demonstrated that some adrenal tumors in man are responsive to ACTH(7). Cohen *et al.* have studied adrenal tumors in mice and rats which respond *in vitro* to ACTH by increased steroid synthesis(5). Plasma ketosteroid studies should be helpful in distinguishing between responsive tumors of the type studied by these workers and tumors such as the type under present study which are not responsive to ACTH stimulation or suppression. The long term effects of ACTH, gonadotropin, and steroid hormones

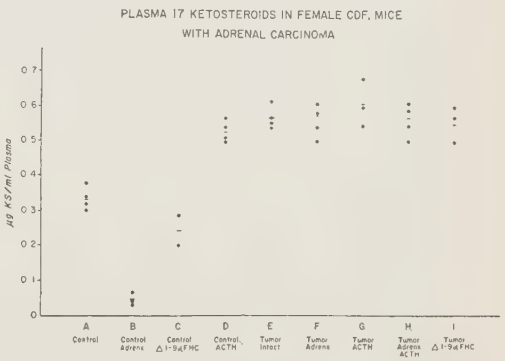

FIG. 2. Adrenalectomy was performed on groups of control and tumor-bearing mice after 20 days of tumor growth in the latter group. ACTH was administered where shown, 1 mg daily for the last 5 days before sacrifice. Δ1-9αFHC was administered where shown, 0.1 mg daily subcut. in oil, for the last 7 days before sacrifice. Control and tumor-bearing animals were sacrificed after 25 days of tumor growth in the latter group. Each point represents a plasma ketosteroid determination on a pool from 4 mice.

on the growth and behavior of this tumor are under investigation. These will be correlated with plasma ketosteroid and corticoid levels. If it could be demonstrated that chemotherapeutic agents affected growth and steroid biosynthetic capacity in a similar fashion in this tumor, the study of plasma steroid response to therapeutic agents may prove to be a useful screening technic.

Summary. A transplantable adrenal mouse carcinoma produced elevated plasma ketosteroid in the host. Non-tumor-bearing mice subjected to gonadectomy-adrenalectomy have negligible levels of plasma ketosteroid. Tumor-bearing mice have unchanged plasma levels following this procedure indicating steroid production by the tumor. ACTH and Δ1-9α fluorohydrocortisone have no influence on the plasma ketosteroid level of tumor-bearing animals. Distant metastases were found in adrenalectomized-gonadectomized males and adrenalectomized females, but not in animals not subjected to these procedures.

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Inhibition of Glucose Uptake by Acidosis *in vitro*.* (23833)

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In diabetic acidosis there is a relative insensitivity to insulin(1). Chari(2) has concluded that a high concentration of acetoacetate is probably one of the factors causing a derangement of carbohydrate metabolism. The work of Guest *et al.*(3) shows however, that the excess hydrogen ion itself is an extremely important factor. They induced a simple ammonium chloride acidosis in dogs and demonstrated a reduced insulin sensitivity and a less favorable glucose tolerance. Guest attributes these effects to an inhibition of hexokinase at the lowered pH. Comparable experiments were conducted *in vitro* to determine whether extra-hepatic tissues were involved.

Method. Hemidiaphragms from rats weighing 150 $\pm$ 10 g were incubated at 38°C in Krebs-Ringer solutions buffered with bicarbonate-CO_2 at pH 7.4 and 6.8. Each solution contained 250 mg/L glucose and some insulin, and was equilibrated with 5% CO_2 in O_2. The glucose uptake of each hemidiaphragm was determined by measuring the disappearance of glucose from 2 ml of the nutrient solution in 1 hour. The glucose determinations were by the anthrone method(4).

Results. When the insulin concentration exceeded 0.08 i.u./ml the glucose uptake was the same at pH 7.4 as at 6.8, but with decreasing insulin concentrations the muscles at the lower pH took up significantly less glucose. With 0.008 i.u./ml insulin the difference was 25%, and it was 80% when no insulin was added to the nutrient medium. The absolute glucose uptake decreases with decreasing insulin concentrations so that the percentage differences become large. Table I lists both the percentage differences and the absolute differences in g/kg/hr at each insulin concentration. The latter are shown graphically in Fig. 1. From these results it may be concluded

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TABLE I.

Units of insulin conc., i.u./ml	No. of pairs	% difference of glucose uptake	Absolute difference of glucose uptake, g/kg/hr
0	6	80.6 ± 10.9	.434 ± .081
.008	8	25.8 ± 5.0	.379 ± .073
.016	6	20.6 ± 2.6	.307 ± .038
.032	6	25.8 ± 3.9	.306 ± .043
.048	8	7.5 ± 4.5	.102 ± .063
.080	6	1.8 ± 7.1	.032 ± .129
.160	18	4.1 ± 2.9	.062 ± .122

$$\% \text{ difference} = \frac{\text{uptake at pH 7.4} - \text{uptake at pH 6.8}}{\text{uptake at pH 7.4}} \times 100.$$

that acidosis will inhibit glucose uptake in the rat diaphragm and that the effect of the acidosis can be overcome by massive increases in the insulin concentration.

At an insulin concentration of 0.008 i.u./ml there is a suitably consistent inhibition of glucose uptake at a lower pH so that the effect of various degrees of acidosis can be studied. The difference of glucose uptake between paired muscles incubated at pH 7.4 and 7.1 was almost as great as that between pH 7.4 and 6.8. There was no enhancement

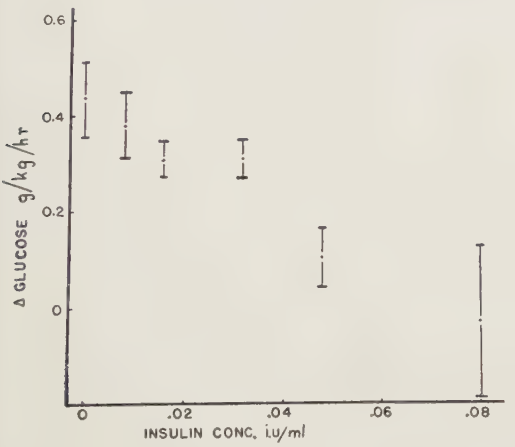

FIG. 1. Inhibition of glucose uptake at pH 6.8 is expressed as the difference between uptake at pH 7.4 and 6.8. This is plotted against insulin conc.

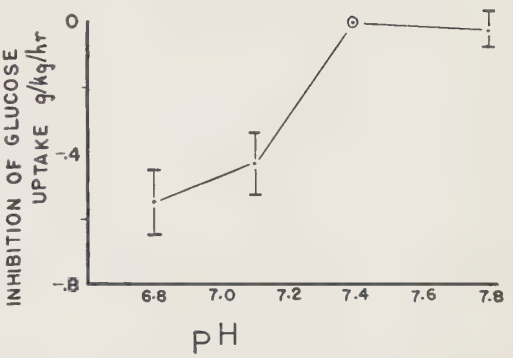

FIG. 2. Effect of pH changes on glucose uptake. Insulin conc. is constant at 0.008 i.u./ml.

of glucose uptake by raising the pH to 7.8 (Fig. 2). At this same insulin concentration, the difference of glucose uptake was the same whether the pH was lowered to 6.8 by the addition of hydrochloric acid or of acetic acid. If it is presumed that the acetate ion penetrates the cell more readily than does the chloride ion, then the similarity of effect on glucose uptake of these two acids suggests that the acidosis affects a surface process and not an intracellular process.

Summary. Glucose uptakes of rat diaphragms *in vitro* were compared at pH 7.4 and 6.8. The acid medium inhibited glucose uptake but the degree of inhibition could be decreased by adding insulin to both media. At insulin concentrations in excess of 0.08 i.u./ml, glucose uptake was the same at each pH. With 0.008 i.u./ml insulin a pH of 7.1 was almost as inhibitory as pH 6.8. There was no enhancement of glucose uptake at pH 7.8.

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Absorption of Cortisol from the Colon in Ulcerative Colitis.*† (23834)

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The successful use of topical steroid therapy for diseases of the skin, eye, and joints has led us to evaluate the effect of cortisol by rectum in iodiopathic ulcerative colitis. Truelove(1) and Allodi and Muratori(2) almost simultaneously reported good clinical results with cortisol enemas, but whether this was the result of systemic absorption of the steroid or a purely local effect was undetermined. Our experience with the clinical efficacy of this form of treatment will be reported in detail elsewhere; the present study is concerned with the absorption of rectally administered cortisol in ulcerative colitis.

Methods. A. *Mode of Administration*: Patients with ulcerative colitis of one to 4 years' duration, proven by sigmoidoscopy and x-ray, received daily enemas at about 10:00 a.m. of 100 cc. of normal saline for a period of 7 to 10 days prior to the administration of cortisol by rectum. After this control period, 200 mg of cortisol (hemisuccinate-sodium) dissolved in 100 cc of normal saline was administered as a rectal enema for a 20-minute period, at the rate of 3-5 ml per minute from an infusion bottle. Daily therapy with the steroid was continued for 2 weeks. Three patients received cortisol-4-C^{14} in a dose of 4,400,000 counts per minute (494 μg) as a tracer dose added to the 200 mg carrier cortisol.

B. Blood samples were obtained at 0, ½, 1, 2, 4, 6, and 24 hours after the administration of saline and cortisol enemas. The levels of plasma cortisol were measured quantitatively in duplicate on the initial day of therapy by the method of Bondy, *et al.*(3) and the presence of conjugated steroids was determined by the method of Cohn and Bondy (in preparation). In these experiments where cortisol-4-C^{14} was employed conjugated steroids were initially separated by paper electrophoresis of a butanol extract of plasma. The conjugated steroids were eluted with 70% methanol taken to dryness *in vacuo* and counted as the total conjugated glucuronide fraction in a gas flow windowless counter, shielded by steel, with a background of 1.8-2.0 counts/minute. Unconjugated cortisol-4-C^{14} was counted in a liquid phosphor scintillation counter (TMC Model LP-2).

Results. Table I illustrates the plasma

TABLE I. Plasma Levels of Cortisol.

Patient	Enema	Hr after enema: 0	½	1	2	4	6	24
S. F.	200 mg cortisol	7.3		3.4	1.4	12.7		4.9
L. D.	.85% saline*	28.5		21.8	16.2	44.2		18.2
	200 mg cortisol	18.2		24.0	37.6	49.5		21.4
M. I.	.85% saline*	12.1	10.0	13.1	13.2	9.4	14.4	15.1
	200 mg cortisol	15.1	18.5	18.2	16.3	24.5	25.3	17.2
P. K.	200 mg cortisol	10.1		17.3	9.0	21.1	14.8	11.2
A. K.	*Idem*	9.4	9.1	13.5	10.2	7.5		4.3
Mean of diff. from zero time conc. (μg/100 ml)				3.3	2.9	11.0		− .2
Upper confidence limit (95%)				8.6	14.9	26.4		4.1

* Statistics do not include cortisol values after saline.

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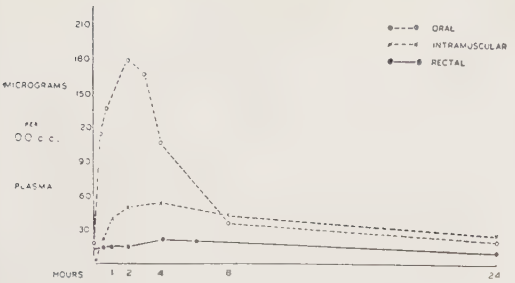

FIG. 1. Avg plasma cortisol concentrations after administration of 200 mg of cortisol.

levels of cortisol. There is a wide range of variation of these levels and no statistically significant differences occurred in the various time periods studied. The 95% confidence limits, assuming a normal distribution of plasma cortisol values(4), are also indicated.

Fig. 1 graphically illustrates the average plasma level radioactivity of cortisol after oral, intramuscular, and rectal administration. The data for oral and intramuscular levels were obtained from Peterson *et al.*(5), whose plasma values are similar to those reported from this laboratory. Peterson's data were obtained from healthy subjects.

Fig. 2 illustrates the levels of radioactive cortisol and conjugated steroids in plasma after the rectal administration of cortisol-4-C^{14} and carrier cortisol (200 mg). The radioactivity of the cortisol fraction is not significantly above the background. The peak of the radioactive conjugated steroids at one hour, however, is significant and is being further investigated.

Discussion. Five patients with idiopathic ulcerative colitis received 200 mg of cortisol daily for a period of 2 weeks. Clinical improvement was noted by sigmoidoscopy and the remission of symptoms. This may have been, in major part, a local effect since there were no statistically significant differences in the levels of plasma cortisol at the various time periods studied when compared to the zero time. In addition, there was no observed clinical evidence of a hyperadrenocortical state during the administration of the steroid.

The levels of plasma cortisol after a saline enema in patient L.D. are similar to those obtained after the cortisol enema. The levels in both instances are above the normal values for our laboratory (7-15 μg/100 ml)(3). It is possible that the zero-hour value of 28.5 μg/100 ml may be a reflection of the effect of the disease on the adrenal cortex, since patients with a variety of serious diseases may have abnormally high levels of cortisol (unpublished data).

The plasma cortisol concentration in normal patients reaches a peak 2 hours after the administration of 200 mg of oral cortisol; a plateau is obtained between 2 and 4 hours after intramuscular cortisol(4). Patients with ulcerative colitis who received 200 mg of rectal cortisol did not demonstrate any significant peak.

Within the first hour the plasma contained conjugated steroids derived from the cortisol given by rectum, but the concentration of these conjugates fell rapidly to non-detectable limits within 4 hours. This indicates that whatever cortisol is absorbed is rapidly metabolized by the liver into the reduced glucuronisides of cortisol, and subsequently excreted in the urine. An increased twenty-four hour excretion of 17-hydroxycorticosteroids and 17-ketosteroids after cortisol enemas has been demonstrated by Nabarro *et al.* (6).

Present knowledge does not permit the statement that the levels of plasma cortisol observed after therapeutic enema are pharmacologically ineffective; however, this was suggested clinically in our study. Complete metabolic balance studies are being conducted which may lend support to our clinical impression that this method of administration is relatively devoid of any of the known side effects of steroid therapy and may therefore

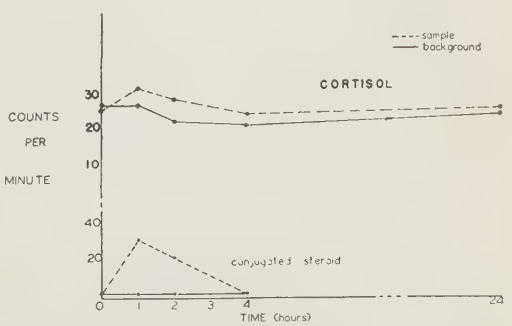

FIG. 2. Plasma steroid radioactivity concentrations after rectal administration of cortisol-4-C^{14}.

be particularly useful in the treatment of acute early ulcerative colitis.

Summary. Rectal cortisol has been used in treatment of chronic ulcerative colitis with good results. Following administration of 200 mg of the steroid dissolved in 100 cc of normal saline, there was no significant increase in blood levels of cortisol. This was confirmed by absence in the plasma cortisol fraction of any appreciable radioactivity following instillation of cortisol-4-C^{14}. By implication, our data suggest that the good clinical results obtained were probably the result of a local effect of the cortisol on the rectal mucosa.

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Fluoride Content of Urinary and Biliary Tract Calculi.* (23835)

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In connection with an extensive survey of the fluoride content of human skeletal tissues (1,2), it was of interest to assess the relation of water-borne fluoride to the fluoride content of urinary and biliary tract calculi. With the exception of the recent duplicate reports‡ by Herman(3) and Spira(4), no data are available on the fluoride content of urinary tract calculi. Murray(5) reported that kidney stones contained fluoride, but no data were given. Data on the fluoride content of biliary calculi appear to be limited to the recent report of Spira(6). In the present study, the fluoride content of urinary and biliary tract calculi was also compared with that of bone obtained from individuals residing in low (0.0-0.6 ppm in the drinking water) and high fluoride areas (2.6 ppm in the drinking water).

Methods and materials. Thirty-three urinary and 9 biliary tract calculi obtained from individuals with verified histories of fluoride exposure were dried overnight at 105°C and pulverized. They were then extracted with alcohol for 8 hours and with ether for 4 hours, ashed at 550°C for 3 hours and analyzed for calcium(7), phosphorus(8) and fluoride(9,10,11). Samples of dry, fat-free urinary tract calculi were submitted for X-ray diffraction analysis to determine if calculi with strong apatite patterns showed higher concentrations of fluoride. A pooled sample of bile from four individuals who had resided in Washington, D.C. (1 ppm F in the drinking water) was also analyzed for fluoride. The fluoride content of urinary and biliary tract calculi was compared with that of some 35 bones taken at autopsy from individuals residing in low fluoride areas (0.1-0.4 ppm) and in a high fluoride area (2.6 ppm F). Data on the fluoride content of bones from individuals drinking water containing 0.1-4.0 ppm F will be presented elsewhere(1).

* Urinary tract calculi were kindly furnished by Elmer Belt Urological Group, Los Angeles, Calif., thru courtesy of Lucien A. Bavetta, and by the Glockner-Penrose Hospital, Colorado Springs, Colo. Biliary tract calculi were also furnished by Glockner-Penrose Hospital. We are also indebted to R. W. G. Wyckoff, M. V. Moseley, and D. B. Scott for X-ray diffraction analysis of a number of urinary calculi.

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‡ Since the fluoride data are identical in both reports, these studies do not appear to be independent investigations.

TABLE I. Fat, Ash, Calcium, Phosphorus, and Fluoride Content of Urinary Tract Calculi.*

Years of residence	Fat	Ash	Ca	P	F	Ca/P	Ca/P:F	Ca/F
			%					
			Low fluoride area†					
1					.41			
4	9.3	63.7	26.1	3.9	.27	6.7	24.8	96.7
9	8.0	66.9	26.6	4.1	.27	6.5	24.1	98.5
9	.3	67.7			.10			
12	20.1	6.9	13.9	16.0	.02	.9	45.0	695.0
13	11.8	76.8	20.7	15.2	.12	1.4	11.7	172.5
14	8.6	69.9	28.1	3.7	.41	7.6	18.5	68.5
15					.67			
16	20.0	54.1			.06			
20	14.8	71.4	10.7	19.6	.03	.6	20.0	356.7
23					.24			
28	6.5	62.5			.08			
32	6.2	84.6	29.8	15.9	.43	1.9	4.4	69.3
33	17.2	22.4			.12			
33	12.7	58.6	25.8	1.8	.15	14.3	95.3	172.0
51§	4.2	78.0			.66			
			Elevated fluoride area‡					
1	4.0	80.3	28.9	13.2	.82	2.2	2.7	35.3
2	14.6	59.1	23.2	1.8	.16	12.9	80.6	145.0
3					.65			
4§	2.0	80.7	27.3	17.0	.23	1.6	7.0	116.0
9	7.8	63.7	24.5	4.5	.39	5.4	13.8	62.8
13	11.9	68.5	11.0	11.9	.14	.9	6.4	78.6
14	8.5	62.6	25.9	9.7	.32	2.7	8.4	80.9
14	6.9	64.9			.10			
20	6.9	61.3			.11			
23	0	85.1			1.11			
25	11.4	63.4	1.9	15.9	.03	.1	3.3	63.3
26	11.6	69.3	28.2	8.9	.50	3.2	6.4	56.4
27					1.00			
29	14.5	39.0	13.7	.3	.06	45.7	762.0	228.3
40	5.0	80.7	23.9	16.1	.34	1.5	4.4	70.3
46	3.5	63.2	24.5	.6	.12	40.8	340.0	204.2
53	6.7	79.2	17.6	14.1	.24	1.2	5.0	73.3

* Based on oven-dry wt (overnight at 105°C).

† Washington, D. C., 0.1 ppm F, one individual; Los Angeles, Calif., 0.0-0.6 ppm F, in drinking water. All individuals resided in low fluoride area for at least 10 yr prior to removal of calculi. "Years of residence" refers to last period of residence.

‡ Colorado Springs, Col., 2.6 ppm F, in drinking water.

§ Same individual. Resided in Washington, D. C. 51 yr prior to fluoridation, and 4 yr thereafter when water contained 1 ppm F.

Results. The concentration of fat, ash, calcium, phosphorus and fluoride of urinary tract calculi classified according to water-fluoride exposure of the individual is presented in Table I.

The mean of 0.25% fluoride in urinary tract calculi of individuals from a low fluoride area, expressed on an oven-dry basis, was not significantly different from that of calculi of individuals from an area where the drinking water contained 2.6 ppm fluoride, *i.e.* 0.37%. Herman(3) and Spira(4) reported a range of 0-0.18% fluoride with a mean of 0.05% fluoride. Their values are considerably lower, in most cases, than those reported for all samples of urinary tract calculi in the present study (0.02-1.11% with a mean of 0.31%) and may be attributed, to some extent, to the fact that only part of the calculus was used for analysis.

As shown in Table I, the Ca/P weight ratio varied widely (0.1-45.7) and was unrelated to the fluoride concentration, Ca/P:F (3.3-762.0). In addition, no relation was apparent between the calcium and fluoride concentrations, Ca/F (35.3-695.0).

The concentration of fat, ash, calcium, phosphorus and fluoride in biliary tract calculi is presented in Table II on an oven-dry basis.

TABLE II. Fat, Ash, Calcium, Phosphorus, and Fluoride Content of Biliary Tract Calculi.*

Years of residence	Fat	Ash	Ca	P	F
			%		
2	96.5	2.1	1.2	.3	.002
15	97.1	1.2	.6	.2	"
17	52.5	40.3	15.9	.1	.006
28	92.2	5.2	2.2	.6	.003
29	77.6	15.0	4.3	.1	.004
35	89.5	8.6	4.2	.1	.001
39	94.5	3.4			.000
Unknown†	97.0	.2			"
" †	98.7	.1			"

* Based on oven-dry wt (overnight at 105°C).
† Amarillo, Texas, 1.6-3.2 ppm F in the drinking water from 1949 to 1957; remaining samples obtained from Colorado Springs, Col., 2.6 ppm F in the drinking water.

As expected, the "fat" comprised at least 90% of the calculus in nearly every case. Fluoride was absent, or present in very low concentration. Spira(6) also recently reported the presence of small amounts of fluoride in biliary tract calculi similar to those reported in the present study (Table II). It is interesting to note that the composite sample of human bile contained only 0.1 ppm fluoride. A sample of ox-bile obtained in this study from an animal exposed to high concentrations of fluoride on forage from air borne sources for four years contained 0.6 ppm fluoride. The small amount of fluoride in biliary tract calculi is in keeping with its low concentration in bile. The extremely low calcium and ash content of biliary calculi (Table II) is probably also a factor in their inability to incorporate fluoride.

The data on the fluoride content of urinary and biliary calculi and of bones from similar fluoride areas are summarized in Table III on both an oven-dry and ash basis.

As shown in Table III, the mean concentration of fluoride was significantly higher in urinary tract calculi than in the bones of individuals from both low fluoride ($p<0.01$) and elevated fluoride areas ($p<0.02$). The mean years of residence of those furnishing the urinary tract calculi and bones were 20.1 and 30.4 years respectively.

The higher concentration of fluoride in the urinary tract calculi may be due, in part, to the higher fluoride concentration reported in urine(12,13) than in blood(14). The concentration of fluoride in the urine closely approximates that in the drinking water at equilibrium(12,13). Blood contains about 0.01 ppm fluoride when the drinking water contains 0.1 ppm fluoride and approximately 0.04 ppm fluoride when the water contains 1.0 ppm fluoride(14). Although the calculi showed no significant increment in fluoride with an increase in the fluoride content of the drinking water, a 4 to 5-fold elevation of fluoride was found in the bones when expressed on either an oven-dry or ash basis. This may be attributed in part to growth and metabolic turnover in bone, whereas, urinary and biliary tract calculi may be considered foreign accretions and hence metabolically inert.

Differences in the composition of calculi and bones may also play an important role in their ability to incorporate fluoride. Thus, the major phase of the inorganic portion of bone is presumed to be a type of hydroxyapatite(15,16), whereas, eleven different crystalline compounds have been identified in some 600 urinary calculi by Lagergren(17) by X-ray diffraction analysis. Although an apatite pattern was seen in 7 of 9 calculi ex-

TABLE III. Fat, Ash and Fluoride Concentration* of Urinary and Biliary Calculi, and of Bones of Individuals from Areas Containing Similar Concentrations of Fluoride in the Drinking Water.

	Fat	Ash	Fluoride conc.	
			Oven-dry basis	Ash basis
Low fluoride area (0.0-0.6 ppm F in drinking water)				
Urinary calculi	10.7 ± 7 (13)	60.3 ± 6.1 (13)	.25 ± .05 (16)	.34 ± .07 (13)
Bone	28.3 ± 4.4 (10)	38.0 ± 3.7 (10)	.04 ± .01 (10)	.11 ± .02 (10)
Elevated fluoride area (2.6 ppm F in drinking water)				
Urinary calculi	7.7 ± 1.1 (15)	68.1 ± 3.1 (15)	.37 ± .08 (17)	.42 ± .09 (15)
Biliary calculi	88.4 ± 5.0 (9)	8.5 ± 4.3 (9)	.002± .0007(9)	.10 ± .03 (9)
Bone	35.6 ± 2.6 (25)	34.8 ± 2.1 (25)	.18 ± .01 (25)	.55 ± .04 (25)

* All values expressed as mean ± stand. error. No. in parentheses refer to No. of samples.

amined and was the chief component of four in the present study, no relation could be observed between crystal structure and fluoride concentration.

Summary. Thirty-three samples of urinary and 9 samples of biliary tract calculi from individuals with known histories of exposure to water-borne fluoride were analyzed for fat, ash, calcium, phosphate and fluoride. (1) Concentration of fluoride in urinary tract calculi was significantly higher than that in the bones. (2) Mean concentration of 0.25% fluoride in urinary tract calculi of individuals from low fluoride area was not significantly different from that (0.37%) of calculi of individuals whose drinking water contained 2.6 ppm fluoride. (3) No relation was apparent between the calcium and fluoride concentrations of urinary tract calculi. (4) Fluoride was present in biliary tract calculi in very low concentration (0.000-0.006%).

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Technic for Collection of Thoracic Duct Lymph of Man. (23836)

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Studies of transport of fat *via* the intestinal lymphatic pathway have been of fundamental importance for our understanding of the mechanism of fat absorption. Great progress has been made in this field during recent years due to utilization of new analytical technics and isotopic labelling of fats, and to a new method for collecting chyle in unanesthetized animals over long periods of time(1). Opportunities for studying chyle in the human have been rare and have been confined to patients with abnormal lymph connections as in the classical experiments that Munk and Rosenstein(2) performed on a girl with spontaneous lymph fistula. Subsequently, a number of cases of chylothorax have been observed (*cf.* Fernandes *et al.*(3)). Recently an extensive investigation was carried out by Blomstrand *et al.*(4) in a case of chyluria, where patient was studied under rigidly controlled conditions for 8 weeks, during which time a series of absorption experiments was conducted. Suitable cases such as those described above are extremely rare, and it is therefore desirable to have a method for collection of lymph from humans under standardized conditions. The present report describes a technic of cannulation of the thoracic duct in man, which has permitted collection of lymph of a given subject for periods of 2-5 days. With this technic for introduction of catheter into the thoracic duct it is possible

TABLE I. Clinical Data on 6 Adult Patients Subjected to Cannulation of the Thoracic Duct. None of the cancer patients had clinical evidence of gastrointestinal dysfunction.

Age	Sex	Clinical diagnosis	Scalene node biopsy	Operation after withdrawal of thoracic duct cannula	Total time for cannulation of thoracic duct, days
48	♀	Bronchogenic carcinoma of left lung with advanced node metastasis	+	Inoperable	5
62	♂	Bronchogenic carcinoma of right lung with mediastinal node metastasis and infiltration in pericardium	—	Pneumonectomy + pericardial resection and removal of node metastasis	4
44	♂	Bronchogenic carcinoma in left lung	—	Pneumonectomy + bloc dissection of the mediastinum	4
62	♂	Bronchogenic carcinoma of left lung with advanced lymphogenic spread	+	Inoperable	2
55	♂	Bronchogenic carcinoma in left lung	—	Pneumonectomy	3
47	♂	Non-tropical sprue			5

to collect the major part of lymph flow without ligation of the thoracic duct. With our method the function of the thoracic duct is thus intact after withdrawal of the catheter in contrast to the technic of Bierman *et al.* (5), who ligated and cannulated the thoracic duct in patients with advanced malignancy. The present method has been useful in the study of absorption of different isotopically labelled compounds and for the study of different aspects of lymph flow.

Methods. Cannulation of the thoracic duct with a polyethylene tubing has been performed on patients with diagnosed cancer of lungs. Relevant clinical data are listed in Table I. The diagnosis has usually been made with the aid of bronchoscopy and histopathological examination of biopsy material taken during this procedure. The routine procedure was then to perform a supraclavicular biopsy of the lymph nodes to exclude metastasis. In connection with this minor surgical procedure the thoracic duct is exposed and a plastic cannula inserted. *Operative procedure.* About two hours before operation, the patient is fed a fat-rich meal to get the thoracic duct better visualized. For cannulation of the thoracic duct a polyethylene tubing (PE 90) is used. The left supraclavicular region is prepared aseptically and the region is anesthetized by infiltration with

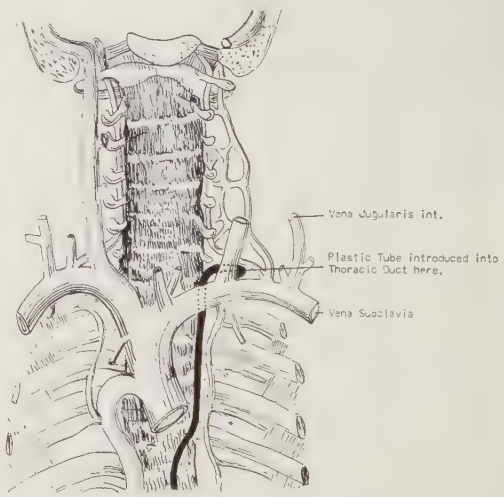

FIG. 1. A schematical drawing to show the anatomical structures involved for the cannulation of the thoracic duct.

xylocain. An incision is made parallel to clavicle and structures involved for cannulation of the thoracic duct are carefully localized (Fig. 1). Some lymph nodes are excised for histopathological examination according to the technics of Daniels(6). The thoracic duct is exposed for 20-30 mm (Fig. 2) by gentle blunt dissection just before its entrance to vena jugularis interna. The thoracic duct is 5-12 mm in diameter and is embedded in connective tissue and fat. A stay suture

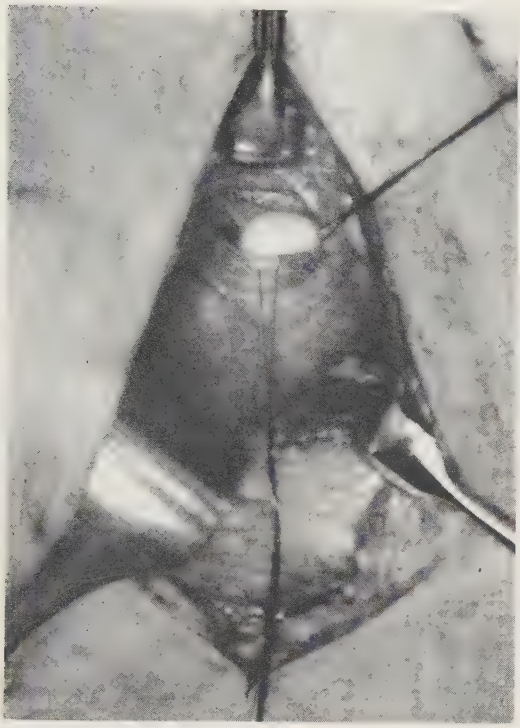

FIG. 2. Photograph of thoracic duct after being prepared free from fat and connective tissue.

is passed into wall of the thoracic duct about 20 mm before its entrance into the vein system. A second suture is placed caudal to the first, and a small longitudinal incision is made in the exposed surface of the thoracic duct close to the stay suture (Fig. 2). A bevelled end of plastic tube is then slipped gently into the duct for 10-20 mm and tied firmly in place with the 2 sutures. The tube is arranged so that it will lie relatively straight in the duct and this can be accomplished by having a suitable bend in the catheter. Before insertion of cannula this is heated and bent in a suitable angle about 10-20 mm from tip. This bend also facilitates introduction of catheter and reduces tension on tube after introduction. By inserting the cannula in the described way it has been possible to collect 100-200 ml of lymph/hour, which is about the same volume as that described by Bierman *et al.*(5) after ligation of thoracic duct cranial to inserted catheter. Apparently gravity drip is enough to drain the major part of lymph flow outside through the plastic catheter, and only a small part of lymph of the thoracic duct is able to reach the vein system. These results thus demonstrate that it is unnecessary to ligate the thoracic duct to collect the major part of the thoracic duct lymph. The present method is apparently a minor surgical procedure compared to complete ligation of thoracic duct and therefore the scope of this operation can be widened to include patients with other diseases than cancer, where it might be expected that the lymph constituents are abnormal. When chyle is not collected the cannula is filled with heparin. In this way we have been able to keep the cannula working for 2-5 days. If aseptic precautions are strictly held there might be no contraindications to collecting thoracic duct lymph for even longer periods as indicated by Bierman *et al.*(5). During the whole time of drainage it is important to control plasma electrolytes and plasma proteins continuously to be able to replace the loss of these substances through the lymph. When the experimental period was ended the wound is partly reopened under local anesthesia. The stay sutures and the catheter were removed and the opening in the thoracic duct is closed with a fine atraumatic catgut suture. In 2 cases after lymph drainage was completed, the tube was sealed and lymph within allowed to clot. The tube was uneventfully withdrawn 2 days later. In one case the catheter was accidentally drawn out during collection of lymph, but the opening in the thoracic duct closed of itself and no leakage was observed.

Biochemical methods: Analyses of the different components of serum and lymph were done mainly according to procedures described earlier(4). The alkaline phosphate activity was determined according to Bessey *et al.*(7), the acid phosphate activity according to Fishman and Lerner(8). Aldolase activity was determined according to Sibley and Lehninger(9). Transaminase activity was measured according to Karmen *et al.*(10), and lactic dehydrogenase was determined according to Wrobleski *et al.*(11).

Results. The thoracic duct has been cannulated in 6 adult patients for 2-5 days. Relevant clinical data are listed in Table I. There was no evidence of clinical dysfunction in any of these subjects during or after drainage

TABLE II. Biochemical Data of Serum and Thoracic Duct Lymph from a Patient with Cancer of the Lungs. The thoracic duct was cannulated with a plastic catheter during local anesthesia and the lymph was collected intermittently for 3 days.

		Fasting serum	Fasting lymph	Lymph obtained during fat absorption
Sodium	meq/l	142	138	140
Potassium		4.7	3.3	4.2
Calcium		4.8	4.4	5.5
Chlorides		98	97	94
Inorg. phosphorus	mg %	2.9	2.7	3.8
Glucose		110	140	120
NPN		29	23	28
Uric acid		4.2	4.1	4.0
Bilirubin		1.0	.8	1.1
Total fat	g %		1.1	5.1
Cholesterol–Total	mg %	207	60	78
Free		57	36	32
Total protein	g %	7.6	5.1	4.1
Paper electrophores	Alb.	4.30	3.42	2.83
	α_1	.34	.26	.22
	α_2	.62	.25	.17
	β_1	.39	.25	.20
	β_2	.29	.15	.11
	γ	1.46	.78	.61
Aldolase	u/ml	6	10	44
Alkaline phosphatase		2.3	1.9	6
Acid prostatic phosphatase		.3	.7	
Transaminase GOT		13	12	12
Lactic acid dehydrogenase		134		173

period. The procedure was well tolerated in all cases. Bowel movements occurred without change. Patients became acclimatised to the cannula very quickly and were ambulatory in the ward.

In Table II are shown some analyses of simultaneously obtained fasting serum and lymph and also lymph on peak of fat absorption during a representative study on a 52-year-old man with bronchial carcinoma of the left lung. The analyses are mainly in accordance with those reported for human thoracic duct lymph by Bierman *et al.*(5). Total cholesterol content of blood was markedly higher than that of lymph. Some interesting observations were made on enzyme concentration of the lymph. Aldolase activity of fasting lymph was higher than that of fasting serum. Some hours later during active absorption the activity increased 4 times. The same phenomenon was observed for alkaline phosphatase. The lactic acid dehydrogenase activity was also higher in lymph than in fasting serum. The acid phosphatase and transaminase did not show any significant difference between fasting serum and fasting lymph. However, if enzyme activity in lymph is corrected for the lower protein concentration both aldolase activity and acid prostatic phosphatase activity are significantly higher than in blood. This phenomenon thus suggests that some enzymes are carried *via* the lymphatic pathway to the blood stream and that it might be of value to determine certain enzyme concentrations in the lymph for diagnostic purposes.

After adding C^{14}-labelled fatty acids to food a prompt appearance of the isotope was observed in cases with normal fat absorption. These results thus clearly demonstrate the usefulness of this method for studying absorption of different labelled fats *via* the lymphatic pathway in man (to be published). One patient with non-tropical sprue has been cannulated and the thoracic duct lymph collected for several days. The results of this investigation will be published elsewhere.

For investigational purposes, a method for collecting thoracic duct lymph in man has long been needed. The present method of cannulating the thoracic duct is a relatively simple procedure to perform on patients in whom for diagnostic and prognostic purposes a supraclavicular lymph node biopsy has to be carried out. No evidence of chylous fistula appeared in any of our cases, nor were any other untoward reactions observed. It is therefore suggested that this cannulation technic might also be used for investigational purposes in other selected patients with *e.g.* malabsorption syndrome, hypercholesterolemia, cancer, hematological disorders, etc. where metabolic disturbances in the lymph constituents can be expected.

Summary. A method for introducing a plastic cannula into the thoracic duct of man is described. The major portion of the thoracic duct lymph can be collected intermittently for 2-5 days. No complications have been encountered. Some preliminary analyses

of human thoracic duct lymph are given. This method has been useful in the study of intestinal absorption of different labelled compounds *via* the lymphatic pathway.

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Tryptophan-Nicotinic Acid Metabolism in Schizophrenia. (23837)

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It is now fairly established that tryptophan is the dietary precursor of 5-hydroxytryptamine(1) which is then oxidatively deaminated by way of 5-hydroxyindolacetaldehyde to yield 5-hydroxyindolylacetic acid(2). The presence of 5-hydroxyindolylacetic acid in normal urine indicates that an appreciable proportion of tryptophan is metabolised *via* 5-hydroxyindole route(3). 5-hydroxytryptamine also occurs in the central nervous system and Woolley and Shaw(4-6) consider it to play a vital part in normal mental processes. They have attributed some naturally occurring psychotic states such as schizophrenia to be the result of an altered metabolism of this compound. Both deficiency as well as excessive formation of serotonin in the brain(4,7) have been held as possible aetiological factors of schizophrenia. 3-indoleacetic acid is also a normal constituent of human urine, but it is not yet settled whether it is a product of metabolism of body tissues or is derived entirely from plant material in diet and bacterial action on tryptophan in the gut(8). In view of these conflicting opinions it was considered worthwhile to study the various metabolic routes of tryptophan in patients suffering from schizophrenia and in normal men.

Material and methods. Twenty adult male patients suffering from schizophrenia admitted in the Lumbini Park Mental Hospital, Calcutta were selected for study. Normal studies were also made on 10 research workers of the laboratory. Their diets did not vary from day to day. The 24-hour urine was collected in bottle containing 10 cc toluene. Urinary excretions of 5-hydroxyindolylacetic acid and 3-indoleacetic acid were determined for 2 consecutive days in 10 subjects of schizophrenia. At end of second day's collection of urine, they were fed 5 g DL-tryptophan and 24-hour urine samples collected daily for 3 subsequent days and analyzed as before. Similar studies were also made on 10 normal subjects. Daily urinary excretions of nicotinic acid, quinolinic acid, N′-methylnicotinamide (NMN), 6-pyridone, tryptophan, kynurenin, anthranilic acid, and 3-hydroxyanthranilic acid before and for 3 days after a similar dose of tryptophan were determined on another 10 patients suffering from schizophrenia. The results of such studies on normal subjects by the authors have already been reported(9). 5-hydroxyindolylacetic acid was estimated chemically by the method of Udenfriend, Titus and Weissbach(3). 3-indoleacetic acid was estimated

TABLE I. Average 24-Hr Urinary Excretions of 3-Indoleacetic Acid and 5-Hydroxyindolylacetic Acid in mg by 10 Patients and 10 Normal Persons.

		Schizophrenia		Normal	
		3-indoleacetic acid	5-hydroxy-indolylacetic acid	3-indoleacetic acid	5-hydroxy-indolylacetic acid
Before feeding tryptophan		17.6 ± 1 *	17.2 ± 1.4	18.5 ± 1.25	7.59 ± .74
Days after feeding 5 g DL-tryptophan	1	48.1 ± 3.46	38.9 ± 3.1	45.3 ± 3.27	9.9 ±1.04
	2	24.7 ± 1.69	20.8 ± 1.3	23.2 ± 1.52	7.8 ± .61
	3	18.2 ± 1.28	17.3 ± 1.2	19.8 ± 1.22	7.54 ± .72

* ± stand. error.

TABLE II. Average 24-Hr Urinary Excretions of Metabolites of Tryptophan and Nicotinic Acid in mg by 10 Patients Suffering from Schizophrenia.

		Nicotinic acid and amide	Quinolinic acid	N'MN	6-pyridone	Tryptophan
Before feeding tryptophan		5.8 ± .44*	11.6 ± .77	5.9 ± .56	5.1 ± .9	0
Days after feeding 5 g DL-tryptophan	1	10.4 ± .44	20.4 ±1.21	14.5 ±1.08	20.4 ± 2.24	169.5 ± 6.64
	2	6.4 ± .46	13.3 ± .95	10.4 ± .88	16.5 ± 2.52	16.1 ± .86
	3	5.6 ± .45	12.1 ± .83	6.5 ± .38	8.6 ± 1.44	0

* ± stand. error.

Kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were absent in all urine samples.

by the method of Gordon and Weber(10). Nicotinic acid, quinolinic acid, NMN, 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were estimated by methods mentioned before(9). The results are given in Tables I and II.

Results. 3-indoleacetic acid and 5-hydroxyindolylacetic acid were present initially in urine of both normal subjects and patients suffering from schizophrenia. After feeding tryptophan urinary excretion of 3-indoleacetic acid increased equally in both groups and gradually decreased on subsequent days. After tryptophan administration, urinary excretion of 5-hydroxyindolylacetic acid rose significantly in patients suffering from schizophrenia but no discernible increase was observed in normal subjects. Nicotinic acid, quinolinic acid, NMN and 6-pyridone were normal urinary metabolites in patients suffering from schizophrenia and all these substances were excreted in higher quantities after tryptophan intake. Tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were absent in urine initially in schizophrenic subjects. Whereas tryptophan appeared in urine for 2 days after feeding, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid could not be detected in urine even after its administration.

Discussion. The increase in excretion of 3-indoleacetic acid after administration of tryptophan both in normal and schizophrenic groups indicates that dietary tryptophan is the precursor of urinary 3-indoleacetic acid; it also suggests that this route of tryptophan metabolism proceeds normally in schizophrenia. Initial high level and greater increase of 5-hydroxyindolylacetic acid excretion in urine after tryptophan feeding in schizophrenia than in normal subjects points to the possibility that rate of serotonin synthesis in this disease occurs at an accelerated rate. The data at hand do not permit the authors finally to conclude about functional ability of amine oxidase which is normally concerned with disposal of serotonin. It is likely that amine oxidase available is not able to cope with the accelerated rate of serotonin synthesis and the consequent accumulation of serotonin is responsible for the mental aberration.

The initial absence of urinary tryptophan and less recovery after its feeding than in normal subjects may be due to its rapid utilisation in other directions. The absence in urine of kynurenine and 3-hydroxyanthranilic acid, which are intermediates in the synthetic pathway of nicotinic acid, after administration of tryptophan in schizophrenia

in contrast to the findings in normal subjects is possibly because of quicker synthesis of nicotinic acid. The high initial urinary nicotinic acid and its greater output after tryptophan feeding in schizophrenia supports this hypothesis.

Summary. 1. Urinary excretion of 5-hydroxyindolylacetic acid and 3-indoleacetic acid was estimated in 10 patients suffering from schizophrenia both before and for 3 days after feeding of tryptophan. Similar studies were made on 10 normal subjects also. 2. Urinary excretion of 3-indoleacetic acid increased both in normal and schizophrenic subjects after administration of tryptophan. Tryptophan also gave rise to increase in urinary excretion of 5-hydroxyindolylacetic acid in schizophrenia but no significant rise was detected in normal subjects. 3. Urinary excretions of nicotinic acid, quinolinic acid, NMN, 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were estimated in 10 schizophrenic subjects before and for 3 days after feeding of tryptophan. 4. Nicotinic acid, quinolinic acid, NMN and 6-pyridone were present in urine initially and they were excreted in greater quantities after tryptophan feeding. Tryptophan which was initially absent also appeared in urine after its administration. Kynurenin, anthranilic acid and 3-hydroxyanthranilic acid could not be detected in urine both before and after administration of tryptophan.

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Purification and Electron Microscopy of Foot-and-Mouth Disease Virus. (23838)

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The present study concerns purification of foot-and-mouth disease virus (FMDV) from infectious tissue culture fluid, as well as identification of the virus particle in the electron microscope by the sensitive method of direct particle counting(1,2) and by aggregation of virus particles by antiviral serum(3). The method of analytical electron microscopy(1) was used to determine the concentration of virus particles in purified virus concentrates and to estimate the number of such particles which constitute one plaque-forming unit (PFU) of infectivity for bovine-kidney monolayer cultures(4).

Materials and methods. Virus was cattle-adapted FMDV, type A, strain 119, obtained from the Research Institute, Pirbright, England. It was subsequently transferred serially 70 or more times in bovine-kidney cultures in Roux flasks, according to procedures previously outlined(5). Twenty to 40 such cultures were used per serial transfer. Each

flask yielded approximately 75 ml of infectious fluid, ranging from $10^{6.5}$ to $10^{8.3}$ PFU per ml. Fluids were harvested, pooled, adjusted to pH 7.5, and stored in 750-ml amounts in paraffin-sealed bottles at –40°C. Smaller volumes for infection of subsequent cultures were placed in flame-sealed ampules. Frozen virus, as needed, was thawed to 0°C. Virus treated in this manner maintained a constant plaque-forming activity for several months. *Infectivity measurements.* Infectious culture fluids and purified viral fractions therefrom were assayed in bovine kidney monolayer cultures by a plaque procedure described in detail elsewhere(4). Twenty to 80 PFU were almost always recorded for one of the dilutions inoculated. In this range twice the standard deviation of plaque counts varies from the mean by less than 31%(4). *Nitrogen analyses.* A digestion and nesslerization procedure with a reproducibility of at least 5% in the range of 5 to 16 μg N per sample was used to determine the protein-nitrogen content of tissue culture fluids and purified virus fractions(1). *Analytical electron microscopy.* Correlation of counts of characteristic physical particles with infectivity and antigenic tests makes unequivocally possible identification of a virus particle in preparations containing only a few particle species (1,3). In these experiments 8 parts of purified virus concentrate in ammonium acetate or ammonium bicarbonate at pH 7.5 were mixed with 2 parts of a 1:1 mixture of 1% bovine albumin (Armour) and uniform-sized polystyrene latex (PSL) spheres (88 mμ and 6.5×10^{10} per ml). The final mixture was deposited on collodion-coated electron microscope specimen grids as microdroplets from the low-velocity spray of a glass nebulizer (6). These droplets after drying, in air and *in vacuo,* to remove volatile and sublimable substances were shadowed with uranium and photographed in the electron microscope. The circular-shaped microdroplet residues were imaged in their entirety at magnifications of 5,000 or greater, sufficient to permit inspection and accurate enumeration of the characteristic particles present. Particle sizes and concentrations were determined by comparison with the known values for PSL spheres. The ratio of physical particles to plaque-forming activity of the purified virus concentrates was then calculated. The critical experiments for identifying the FMDV particle were similar to those of Eckert *et al.*(3) for the identification of myeloblastosis virus particles in which degrees of virus neutralization and precipitation of virus adenosine triphosphatase activity by specific immune serum were correlated with aggregation of particles in the electron microscope. In the present experiments steers were used as the homologous host for preparation of immune sera. After recovery, their neutralizing antibodies were enhanced by suitably-spaced booster injections of virus. In preparation for the particle aggregation tests, sera and purified virus concentrates were mixed as follows: Bovine albumin, usually employed for spraying with virus concentrates and PSL particles, was replaced wholly or in part by serial dilutions of immune serum in the tests and by normal serum in controls. The mixtures were incubated at 37°C for one hour, and portions were then used for electron micrography to determine degree of aggregation of the characteristic physical particles. A measure of aggregation was obtained from the difference between particle concentration in normal serum and concentration of unclumped particles in the presence of antibody. *Virus purification.* Purification and concentration sufficient for imaging and identification of FMDV in the electron microscope were accomplished by successive application of chemical and physical procedures not detrimental to the unstable infective property of this virus(7). Degrees of purification at each stage were established by infectivity and protein-nitrogen assays. The purification and concentration procedure is summarized as follows: Frozen infective tissue culture fluid (320 to 2,400 ml) at pH 7.5 was thawed to 0°C, and the virus, as well as considerable amounts of nonviral protein, was aggregated at –3°C by slow addition of methyl alcohol to a concentration of 20%. The suspension was stored overnight or longer at –6°C and centrifuged at 2,000 rpm for one-half hour in an International refrigerated PR-1 centrifuge. The sedimented precipitate was suspended at pH 7.5 to one-fourth the original

TABLE I. Recovery of Infectivity and Degree of Purification of Type A Foot-and-Mouth Disease Virus.

Purification step No.	Fraction	Recovery of infectivity (%) Tissue culture pool No. 1	2	3	4	5	6	7	8	9	10	avg*	Purification* μg PN/ml	Increase in specific infectivity†
	Original TC fluid											100	230	
1	Soluble part of methanol ppt.	80	69	100	92	92	71	97	100	87	88	88	60	3.4-fold
2	Aqueous phase after extraction with organic solvent (s)	54	69	107	106	116	89,80‡	55	112§	87	95	88	9	22.8-fold
3	Purified virus concentrates after differential centrifugation	27	22	36	62	62	27	30	75	15	82	44	1.5	68.0-fold

* Avg of 10 experiments; μg PN of the fractions were corrected to unconcentrated basis.
† See text for definition.
‡ BuOH-$CHCl_3$ mixture followed by trichlorotrifluoroethane.
§ Trichlorotrifluoroethane instead of BuOH-$CHCl_3$.

volume in phosphate-buffered saline (0.11 M NaCl in 0.01 M Na_2HPO_4) or 0.15 M ammonium acetate. Two volumes of a 50:50 or 85:15 mixture of *n*-butanol and chloroform or, in some experiments, of trichlorotrifluoroethane were introduced with stirring. Mixing was accomplished over a one-half hour period in the former case by intermittent shaking and in the latter case by a Servall Omnimixer operating at top speed with its cup immersed in an ice bath. The organic aqueous emulsion which resulted was broken by centrifugation at 2,000 rpm for one-half hour. The separated aqueous phase contained the virus. Further purification and concentration were accomplished by one cycle of high-and-low-speed centrifugation in a Spinco Model-L centrifuge. The virus was sedimented from the aqueous phase during a 135- or a 150-minute run in a No. 30 rotor at 30,000 rpm (78,410 g). The resulting virus pellet was suspended by vigorous pipetting in a small volume of phosphate-buffered saline, ammonium acetate, or ammonium bicarbonate at pH 7.5 and finally cleared of large insoluble aggregates by centrifugation in a No. 40.2 rotor at 10,000 rpm (6,370 g) for 30 minutes. The supernatant fluid from this run represented the purified virus concentrate and, when in ammonium acetate or bicarbonate, was ready for electron microscopy. Purified concentrates in phosphate-buffered saline were suitable for protein-nitrogen analyses, but for use in electron microscopy were first dialyzed against ammonium acetate. The degree of concentration achieved varied between 50- and 400-fold depending upon the initial volume of infectious tissue culture fluid and upon fluid volumes used to resuspend high-speed pellets. The temperature was maintained at 4°C except where otherwise stated.

Results. Recovery and purification of virus infectivity in 10 experiments are summarized in Table I. The average recovery of virus after successively applied purification steps of alcohol precipitation, extraction with organic solvents, and differential centrifugation was 88, 88, and 44%, respectively. Infectious tissue culture fluids averaged 230 μg of protein nitrogen per ml (μg PN/ml). Corrected to an unconcentrated basis, the successive fractions contained on the average 60, 9, and 1.5 μg PN/ml. Therefore, the increase in specific infectivity of the virus for each step, *i.e.*, equivalent to increases in infectivity to protein-nitrogen ratios, was 3.4-, 6.7-, and 3.0-fold, respectively, the overall increase being 68-fold. If, however, infectivity was lost without concomitant disruption of virus particles, then virus-particle purification would be 153-fold. It is known that this can occur since 22-mμ particle counts (see below) of purified virus concentrates stored for several weeks at 4°C remained constant, while

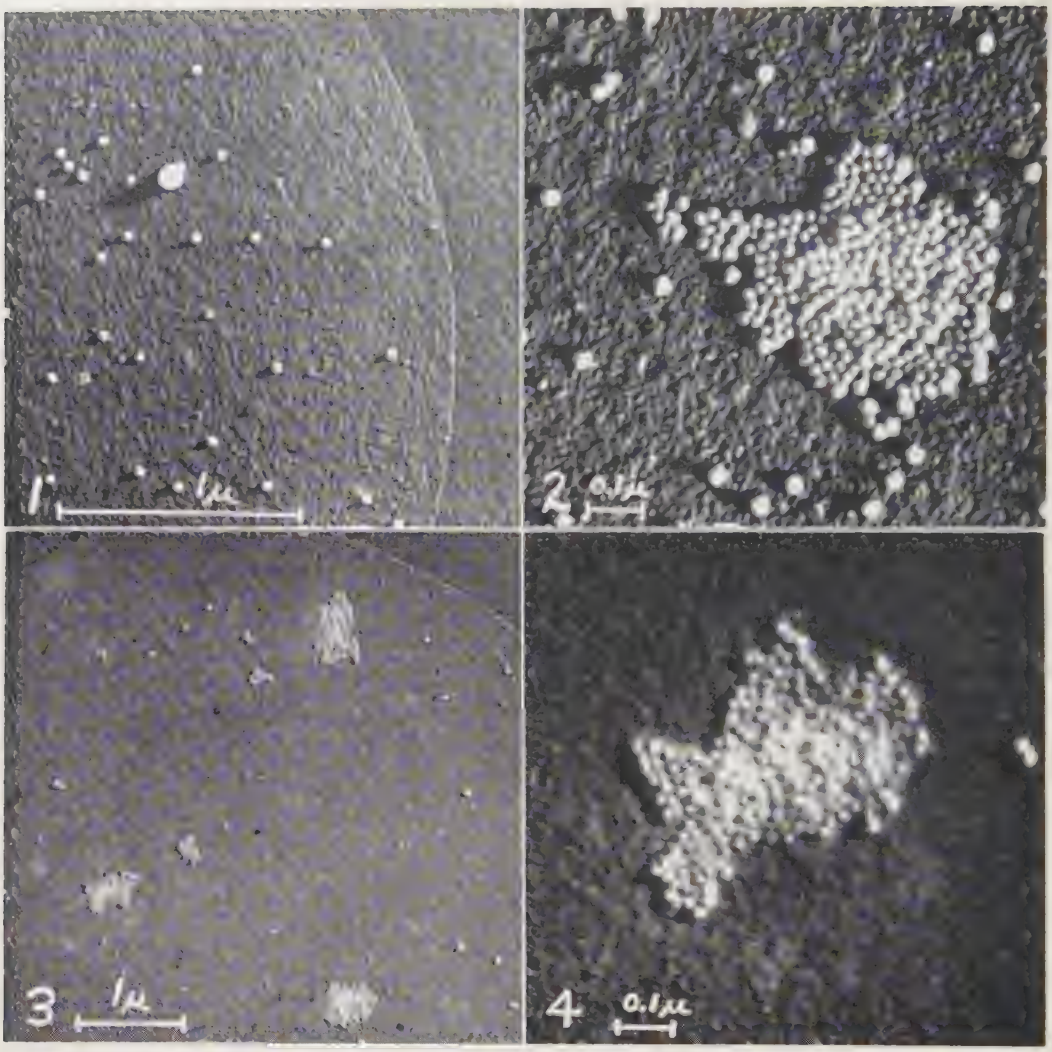

FIG. 1. Section of spray droplet with 22-mμ FMDV particles, normal bovine serum albumin, and a single 88-mμ polystyrene latex sphere, × 31,200.

FIG. 2. Cluster of 22-mμ FMDV particles without bovine serum albumin, × 65,000.

FIG. 3. Section of spray droplet showing 22-mμ FMDV particles specifically aggregated in the presence of immune bovine serum, × 13,000.

FIG. 4. FMDV aggregated with immune bovine serum, × 65,000. Here and in Fig. 3 the particles, while still distinguishable as the 22-mμ type, appear enmeshed in matrices derived from immune bovine serum components.

infectivity decreased to about 0.01% of its initial value.

Electron micrographs of air-dried spray-droplet patterns derived from purified virus concentrates revealed a uniform-sized, discrete particle in large numbers (Fig. 1) not found in control concentrates prepared from the fluids and extracts of comminuted cells of uninfected tissue cultures. When measured individually, this particle had an apparent width of about 28 mμ, but in linear groupings of clusters which sometimes form in the absence of albumin the average width approaches a minimum value of 22 mμ (Fig. 2). The latter value may approximate the true dimension of this particle since the 28 mμ diameter of poliovirus in close-packed arrays is consistent with its diameter in frozen-dried preparations where flattening due to surface-tension forces does not occur(6). The term

"width" rather than "diameter" is used since contours of air-dried 22-mμ particles and their shadows suggest a roughly spherical particle with angular surface. Whether the observed angularity resides in the particle itself or is an artifact due to asymmetrical flattening or accretion of foreign material remains to be determined on frozen-dried specimens. For example, Kaesberg(8) and Williams(9) have shown by precise analysis of particle and shadow contours that certain so-called spherical plant and insect viruses are actually icosahedrons.

The 22-mμ particle content and infectivity of purified virus concentrates were correlated. The two kinds of measurement were made with nearly the same precision. In plaque assays of FMDV infectivity the standard deviation is about 15% when 47 to 66 plaques are counted(4). A comparable standard deviation in the ratio of 22-mμ particles to PSL spheres, *i.e.*, 14%, is obtained by counting as few as 50 of the PSL spheres randomly mixed with a large excess of 22-mμ particles(1). Four times the minimum number of PSL particles were counted, and the 22-mμ counts usually exceeded the PSL by 5- to 50-fold. All particles of both types in a droplet pattern were counted. Indication of random mixing at the time of spraying was obtained from ratios in undiluted, 2-fold, and 4-fold dilutions of a purified virus concentrate. The respective ratios of 22-mμ particles to PSL spheres were 5.3:1, 2.4:1, and 1.15:1. The 22-mμ particles could be counted at concentrations as low as $10^{9.7}$ per ml by examining many droplet patterns; counting was easier when concentrations were 10^{11} or higher. Depending upon individual infectivities of tissue-culture-virus pools, which ranged from $10^{6.5}$ to $10^{8.3}$ PFU per ml, and loss of particles and infectivity during purification and concentration, the average 22-mμ particle/PFU ratio for 11 purified virus concentrates was 690 (Table II). The spread was wide, ranging from 140 to 1,600 physical particles per PFU in experiments where the *n*-butanol-chloroform mixture or trichlorotrifluoroethane was used alone. In one case where both were used successively this ratio was only 33. Therefore, presumptive but not conclusive evidence based on particle counting alone was obtained for identity of FMDV with the 22-mμ particle. Convincing evidence would be a relationship of one physical particle per minimum infectivity unit.

TABLE II. Recovery of Infectivity, 22-mμ Particles, and Ratios of Particles to Plaque-Forming Activity in Purified Concentrates of Type A Foot-and-Mouth Disease Virus.

Purified virus concentrate No.	Recovery of infectivity (%)	22-mμ particles/ml	PFU/ml	22-mμ particles/PFU
5	62	9.7×10^{10}	8.0×10^{7}	1200
6*	27	6.5×10^{9}	2.0×10^{8}	33
7	30	2.5×10^{11}	$3.1 \times$ "	800
8†	75	5.4×10^{10}	7.5×10^{7}	720
9	15	5.2×10^{9}	$2.7 \times$ "	190
10	82	2.5×10^{11}	7.0×10^{8}	360
11	71	8.5×10^{10}	$3.5 \times$ "	240
12	30	$2.6 \times$ "	3.2×10^{7}	810
13	55	$8.4 \times$ "	$5.5 \times$ "	1500
14	40	4.8×10^{11}	3.0×10^{8}	1600
15	11	1.2×10^{10}	8.5×10^{7}	140
			Avg	690

* BuOH-$CHCl_3$ mixture followed by trichlorotrifluoroethane.

† Trichlorotrifluoroethane instead of BuOH-$CHCl_3$.

Since this 1:1 relationship was not found and because another particle approximately 8 mμ in diameter was also seen in electron micrographs of some purified virus concentrates, evidence of an unequivocal nature for the identity of the 22-mμ particle uniquely present in purified virus concentrates was sought and obtained. It was found by analytical electron microscopy that the 22-mμ particles aggregated into clumps when treated with serum from steers immunized with FMDV derived from bovine-tongue epithelium (Fig. 3 and 4). Aggregation was specifically due to the antiviral serum and did not occur in the presence of normal bovine serum. Moreover, diminution of particle aggregation corresponded approximately to the degree of dilution of the immune bovine serum. Unlike the individually resolved particles of Fig. 2 prepared without added serum or serum albumin, particles within aggregates in the presence of immune serum were poorly delineated probably because of accretion of antibody molecules. The occasional clustering of some of the 22-mμ particles which oc-

curred in the absence of added serum albumin (Fig. 2) differs both in kind and degree from the aggregation brought about by immune bovine serum.

Discussion. In contrast with earlier unconvincing attempts by von Ardenne and Pyl (10), Bernard *et al.*(11), and Epstein *et al.* (12), results of the present studies clearly establish that the 22-mμ particles are the physical agents of foot-and-mouth disease and that, depending upon viability, the number of virus particles constituting one PFU for bovine-kidney monolayer cultures varies from 33 to 1,600. This wide variation may be attributed in part to the lability of FMDV infectivity(7) and also to possible insensitivities of the plaque assay in bovine-kidney cultures. Physical particle to infectivity ratios lower than 33:1 for FMDV may be difficult to obtain, especially since this value approximates the minimum ratio of 36:1 found for the more stable poliovirus on sensitive human amnion cells(13,14).

The 8-mμ particle can be eliminated as being virus on the basis that it is not always present in purified concentrates, and also that it cannot be associated with a sedimentation constant, s_{20}, of 137 S found for FMDV by Bachrach(15) and later confirmed at 142 S and 140 S by Randrup(16) and Pyl(17), respectively. An s_{20} value of this magnitude, while completely inconsistent with an 8-mμ particle, would be expected for a 22-mμ virus nucleoprotein in which the nucleic acid is an appreciable portion of the total mass of the particle, possibly as high as one-third of the mass as is the case with poliovirus(18). It has not been the purpose of the present study to identify the 8-mμ particle. It may be the extraviral complement-fixing antigen first observed in centrifugation experiments by Traub and Pyl(19) and later by Bachrach(20) and Bradish *et al.*(21).

Several methods of virus purification were used without success. Physical adsorption methods, protamine-sulfate clearing, and alternate freezings and thawings added little to the degree of virus purification, while enzyme treatments with ficin, papain, trypsin, ribonuclease, and desoxyribonuclease destroyed FMDV infectivity.

On the basis of the 22-mμ size and concentration of FMDV as determined by electron microscopy, the amount of protein and nucleic acid nitrogen attributable to virus may be calculated, assuming that about one-sixth of the particle weight is nitrogen. When this value is compared with protein-nitrogen values determined by digestion and nesslerization of purified virus concentrates, it is found that the 22-mμ particles constitute no more than 1% of the total protein of purified concentrates. This residuum of protein of low molecular weight is being effectively eliminated by zone centrifugation in density gradients and will be the subject of another paper.

Summary. Foot-and-mouth disease virus (FMDV), type A, from bovine-kidney tissue cultures has been purified successively by methanol precipitation, extraction with organic solvents, and differential centrifugation. The FMDV particle in the resulting purified virus concentrates has been identified by analytical electron microscopy in air-dried specimens as a uniform-sized 22-mμ particle. This identification was made through correlative experiments relating particle counts to infectivity and to aggregation of the particles with antiviral bovine serum. While an average of 690 virus particles was present in one plaque-forming unit for bovine-kidney monolayer cultures, a value as low as 33 was obtained.

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Influence of Vit. B_{12} and Sulfur Amino Acids on Liver Glutathione Level in Mice. (23839)

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Several recent reports suggest the existence of a relation between Vit. B_{12} and sulfhydryl metabolism. Dubnoff showed this vitamin to play a role in the reduction of S-S groups(1). Ling and Chow(2) found low non-protein sulfhydryl values in rat erythrocytes deficient in cobalamin, while Register(3) reported low levels in both erythrocytes and livers in B_{12}-deficient rats as compared to the corresponding control animals. In previous studies, we observed low values of reduced glutathione (GSH) in erythrocytes of rats, raised on a diet deficient in Vit. B_{12}(4); nonetheless, liver levels in both rats and mice kept under these conditions were normal(5,6). In Sanguinetti and Marchetti's experiments, the liver GSH in B_{12}-deficient rats was even higher than in the controls(12).

In the present paper the effect of Vit. B_{12} and sulfur amino acids on the GSH level of livers of mice is further studied.

Materials and methods. 300 adult albino mice of our stock were used. Those used for B_{12}-supplemented groups had been kept on complete commercial rat stock ration containing about 3 μg/100 g of this vitamin. Those deficient in Vit. B_{12} had been raised for several generations on a soybean-corn ration (diet 1, Table I). The performance of this deficient stock has been studied in detail(7). All animals used were between 10 and 14 weeks old when the respective experiments were started. At this age, the large weight differences existing at weaning between the B_{12}-deficient and normal mice have practically disappeared, all weighing between 22-25 g. The mean weight at weaning at 28 days of the deficient animals was 12 g, while that of the controls was 17 g. Care was taken that all B_{12}-deficient mice had a weaning weight between 11 and 13 g and continued on the deficient diet until the start of the experi-

TABLE I. Composition of Experimental Diets.†

No.	Soy meal	Bean meal	Corn meal	Starch	Sesame oil	Salts USP XII No. 2	Vit. mixture*
1	46		46		5	2	1
2		50		42	5	2	1
3				92	5	2	1

* Containing/100 g of diet: Thiamine-HCl, 0.3 mg; riboflavin, 0.3 mg; pyridoxine-HCl, 0.2 mg; Ca-pantothenate, 0.2 mg; niacin, 2 mg; folic acid, 0.025 mg; biotin, 0.01 mg; p-aminobenzoic acid, 25 mg; inositol, 10 mg; choline-HCl, 100 mg.

† 0.2% of percomorphum oil and 0.2% of wheat germ oil added.

TABLE II. Liver Glutation in Adult Mice Kept on Different Experimental Diets with and without Vit. B_{12}.

No.	Exp. diet	Days on diet	Not deficient* No. of animals	Not deficient* Liver GSH, mg/100 g	Deficient† No. of animals	Deficient† Liver GSH, mg/100 g
1	Stock	Since birth	15	267 ± 12 ‡		
2	No. 1 Soy bean-corn	*Idem*			13	266 ± 9 ‡
3	*Idem* + 3 μg/100 g of B_{12}	"	15	256 ± 7		
4	No. 2 Methionine-low	30 days	18	72 ± 2.3	25	89 ± 2.4§
5	*Idem* + .3% d,l-methionine	30	16	256 ± 10	20	244 ± 9
6	No. 3 Protein-free	1	5	129 ± 12	5	111 ± 4
7	*Idem*	2	8	99 ± 6	4	97 ± 7
8	No. 2 Methionine-low	1	12	88 ± 6	12	72 ± 2.3‖
9	Fasting	1	6	175 ± 7	11	151 ± 6 ¶
10	"	2	7	175 ± 22	8	165 ± 10
11	No. 3 Protein-free	4	18	89 ± 7	20	68 ± 5 **
12	*Idem* + .25% l-cysteine HCl	4	7	206 ± 22	14	218 ± 11
13	" + .2% l-cystine	4	7	241 ± 17	21	234 ± 14
14	" + .25% d,l-methionine	4	8	190 ± 17	5	212 ± 14

* Animals raised and kept on complete stock ration until start of exp.
† " " " " " B_{12} deficient soy bean-corn ration until start of exp.
‡ Stand. error of the mean.
§ Difference from corresponding B_{12} supplemented control group significant; $t = 5.0$, $p < .01$.
‖ *Idem* $t = 2.5$, $p = .02$.
¶ " $t = 2.8$, $p < .02$.
** " $t = 2.4$, "

ments. Other deficiency symptoms include delayed maturity and high mortality of litters. In diet 2, cooked, dried, and ground black beans (*Phaseolus vulgaris*) were used as the protein source, as these pulses are low in methionine and adequate in all other essential amino acids(8). Liver GSH was determined in duplicate by a modification(6) of the method of Grunert and Phillips(9) and the results were calculated as mg of reduced glutathione (GSH)/100 g of fresh tissue, although it is understood that they rather represent the non-protein sulfhydril (NPSH).

Results. In the first 3 series of Table II are presented the results of experiments on the influence of Vit. B_{12} on GSH levels in mice kept since birth on soy bean-corn rations with or without added Vit. B_{12}, or on a stock diet. No differences between experimental groups could be found in the liver values (Table II, Nos. 1-3).

The liver GSH values of animals eating the methionine-low bean diet, supplemented with methionine, were normal, whether or not Vit. B_{12} was added (Table II, No. 5). When rats were fed the methionine-deficient diet without B_{12} for one month, they had significantly higher liver GSH values than the corresponding group, which had been supplemented with B_{12} (Table II, No. 4).

In other experiments normal and B_{12}-deficient mice were given a protein-free, or methionine-low diet for 1 or 2 days, or were fasted. The methionine-low diet caused the liver GSH levels to drop more rapidly and to lower values than either the protein-free diet or fasting (Groups 6-10). The last treatment was the least effective in lowering these levels. Comparing the Vit. B_{12}-deficient groups with their respective controls, a tendency can be observed for the former to have lower GSH values than the latter, although these differences were statistically significant only in 2 cases, with the methionine-low, and one of the fasting groups.

In another experiment, the influence of sulfur amino acids on GSH was studied in B_{12}-deficient and normal mice. Only the protein-free diet fed for 4 days caused a significantly greater drop in liver GSH levels in animals deficient in Vit. B_{12} than in the controls. Mice kept on similar rations, but supplemented with cysteine, or cystine, or methionine in equivalent amounts, had nearly normal values, whether or not they were deficient in Vit. B_{12} (Groups 11-14). There was no

significant difference between the diets supplemented with any one of the 3 amino acids in their action on liver GSH.

Discussion. In the present experiments, a Vit. B_{12} deficiency reduced hepatic GSH levels only in mice fed for short periods on the protein-free, or methionine-low diets, or fasted. This suggests that the influence of some stress factor (low intake of sulfur amino acids) may be important in revealing the effect of Vit. B_{12} on GSH in the liver.

Vit. B_{12} had no effect on GSH levels of mice fed an otherwise complete diet. This is evident from the data of Table II. Vit. B_{12} also had no effect on the liver GSH levels of mice fed protein free diets supplemented with sulfur amino acids (Series 10-14, Table II). It is difficult to compare these results with those published by Register(3) who found different GSH levels between B_{12}-deficient and supplemented, but otherwise normal rats, as he used stock animals which were put on a B_{12}-low diet for the experiments, while the controls were injected with large amounts of that vitamin. This fact could produce some influence on food consumption or some other unnoticed effects. In our experiments, all the B_{12}-deficient animals were born from deficient dams, and kept on the deficient diet, while the controls came from normal mothers and had never consumed a B_{12}-deficient diet.

The mice kept for 4 weeks on the methionine-low diet had higher liver GSH levels when they were deficient in Vit. B_{12}, than those which were not (Table II, Group 4). This unexpected observation may be comparable to some results of Register and Bartlett (11) who found that exposure to cold or injection of epinephrine raises the liver GSH of methionine-deficient rats, although these treatments lower the liver sulfhydryl in normally fed animals. Recently, Sanguinetti and Marchetti found liver GSH in B_{12}-deficient rats 35% higher than in the controls (12).

The low values found in mice after the consumption of the bean diet are in accordance with the role of the sulfur containing amino acids on the glutathione level(13) and the low methionine content of these pulses(8). We did not observe the slow rise of liver NPSH in mice kept on sulfur amino acid-low diets from the low values after 24 hr to higher values after 2-3 weeks, described by Beck and Bianconi(14).

The methionine-low diet had the most pronounced effect on liver GSH. After only 24 hours on this ration, the values had dropped to nearly as low a level as after 1 month of the same dietary treatment (Table II Groups 4 and 8); fasting caused a much less significant change, while the effect of the protein-free ration was intermediate. These results are in accordance with the relation of glutathione to carbohydrate metabolism, and would suggest also a role of this tripeptide in protein metabolism.

A relation between Vit. B_{12} and methionine synthesis(15) as well as cystine reduction(16) has been postulated; it was therefore of interest to study the effect of the different sulfur amino acids in glutathione formation in mice consuming the protein-free diet. The results presented in No. 11-14, Table II would not indicate an impaired utilization of cystine, as compared to cysteine or methionine in the B_{12}-deficient animals. Anderson and Stekøl likewise found normal incorporation of cystine into liver GSH in B_{12}-deficient rats(17).

In order to control the deficiency of the B_{12}-low experimental groups, Vit. B_{12} was determined in the pooled samples of livers and kidneys of 6 control and 6 deficient mice of the present experiments (series 1 and 2, Table II), using a *L. leichmannii* method with the results presented in Table III. The differences observed between the deficient and control groups are of the same order as those found in rats(5). The control groups had Vit. B_{12} liver values similar to those found by other authors(18). The liver GSH values of the mice of Groups 1 and 2 in Table II were

TABLE III. Vitamin B_{12} in Livers and Kidneys of Adult Mice.

Diet	Vit. B_{12}, $\mu g/g$, in Livers	Kidneys
B_{12}-deficient*	.152	.92
Control†	.949	2.27

* Weaning wt 12.1 g.
† " " 17.2 g.

similar to those found in rats kept on the corresponding diets although the Vit. B_{12} organ levels in the latter were much lower(5).

The present results indicate that the relation between Vit. B_{12} and reduced liver glutathione is not a simple one and that the effect of the latter on the former is only evident under special experimental conditions.

Summary. No differences were found in liver soluble GSH levels between Vit. B_{12}-deficient and control mice, kept on otherwise normal diets, or on protein-free, or methionine-low rations supplemented with sulfur amino acids. When kept for a short period on diets free of protein, or low in methionine, or fasted, mice deficient in Vit. B_{12} had lower hepatic GSH levels than the corresponding controls. After 1 month on a methionine low diet, these levels were higher in B_{12}-deficient animals than in the corresponding controls. Cysteine, cystine, and methionine were equally effective in maintaining the soluble liver GSH at high levels in B_{12}-deficient or supplemented mice consuming protein-free diets.

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Development of a Possible *in vitro* Assay for Intrinsic Factor.*† (23840)

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Miller, Raney, and Hunter(1,2) reported an enhancement of Co^{60}-labeled Vit. B_{12} uptake by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC), and this finding has been confirmed by Latner and Raine(3), and Herbert and London(4). The latter workers(4,5) reported that enhancement of Co^{60}-B_{12} uptake by HIFC was calcium-dependent, reversible to an appreciable degree by ethylene-diamine-tetraacetate (disodium Versenate®), and occurred in the cold as well as at 37.5°C. Most enhancement by HIFC occurred in first hour of incubation(5). Further studies on the mechanism of the enhanced uptake of Co^{60}-B_{12} by rat liver slices in the presence of HIFC are here reported, along with the development of a possible *in vitro* assay for intrinsic factor

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based on these studies. A preliminary report of this work has been presented(6).

Materials and methods. The incubation medium was Krebs-Ringer solution(7), maintained at pH 7.5 with 0.1 M tris-hydroxy-amino-methane (Tris) buffer(8), and with sufficient added $CaCl_2$ to bring the calcium concentration to 10 mM. Tris buffer was chosen because it does not limit the calcium concentration to the same extent as do phosphate and bicarbonate buffers. Two to three hundred mg of rat liver slices were placed in 20 ml beakers, each containing 10 ml of buffer. All incubations were performed for one hour, at 0°C, in air, with constant gentle shaking. Since the uptake of Co^{60}-B_{12} by rat liver slices without HIFC is lower in the cold than at 37°C(5), cold incubation produces a more striking demonstration of enhanced uptake in the presence of HIFC. On incubation at 37°C, liver slices become increasingly friable with time. Increased friability is accompanied by decreased reproducibility of results. No such increased friability is observed at 0°C. Each incubation was followed by decanting the supernatant fluid and washing slices 3 times in 10 ml aliquots of buffer. Where HIFC or Co^{60}-B_{12} was added prior to incubation, the added agent was first dissolved in one ml of 0.9% NaCl. Control samples had one ml of 0.9% NaCl added. The amount of Co^{60}-B_{12} added was always 0.01 μg (specific activity 1 μcurie/μg). For experiments with *simultaneous* incubation in HIFC and Co^{60}-B_{12}, both agents were added prior to a single incubation. For experiments with *sequential* incubation, only HIFC was added prior to first one hour incubation, and only Co^{60}-B_{12} prior to second one hour incubation. For all experiments, retained radio-activity of washed slices was determined in a well-type scintillation counter. The results were recorded in terms of Co^{60}-B_{12} counts/minute/g of liver slices. All HIFC preparations used were from the same source.§

TABLE I. Co^{60}-B_{12} Uptake by Rat Liver Slices after Simultaneous Incubation in HIFC and Co^{60}-B_{12}.

HIFC preparation	HIFC conc. (μg/11 ml)	Liver slice uptake (counts/min./g)
671A	500	495
	375	661
	250	994
	125	1090
	50	1109
	37.5	1535
	25	1795
	12.5	1257
	5	698
671B	500	684
	375	816
	250	1381
	125	1407
	50	1897
	37.5	1530
	25	1176
	12.5	755
	5	442
None	0	425
	0	363

TABLE II. Co^{60}-B_{12} Uptake by Rat Liver Slices after Simultaneous or Sequential Incubation in 500 μg of HIFC #671B.

1st hr		2nd hr		Liver slice uptake (counts/min./g)
HIFC	Co^{60}-B_{12}	HIFC	Co^{60}-B_{12}	
0	0	0	+	241
+	0	0	+	4900
+	+	*	*	644
+	+	0	+	5510
0	+	0	+	399
0	0	+	+	691
+	0	+	+	595

* No second hr incubation.

Results. Table I demonstrates that for any given preparation of HIFC there was an optimal concentration which produced maximal Co^{60}-B_{12} uptake by rat liver slices. Either increasing or decreasing the HIFC concentration from this optimal level resulted in a decreased enhancing effect on Co^{60}-B_{12} uptake.

Table II demonstrates that rat liver slices incubated in a high concentration of HIFC, washed, and subsequently incubated in Co^{60}-B_{12} take up much more Co^{60}-B_{12} than slices incubated in these materials together. Furthermore, slices which have taken up less Co^{60}-B_{12} because of being incubated in HIFC and Co^{60}-B_{12} *simultaneously* may have their Co^{60}-B_{12} uptake increased to that of *sequentially* incubated slices by undergoing a second incubation in Co^{60}-B_{12} alone.

Table III shows the results of *in vitro* assay of 7 HIFC preparations, as performed by

§ Kindly provided by Drs. W. L. Williams and L. Ellenbogen of Lederle Laboratories, Pearl River, N. Y.

TABLE III. Relative Potencies of 7 HIFC Preparations by *In Vitro* and *In Vivo* Determination.

Preparation	*In vitro*, counts/min./g*	*In vivo*, units of potency
WES655	4676,5095	50
671A	1522,1345	10
671C	1147,1316	7–10
671B	784,874	2
186–1	213,192	1
671F	30,54	½
671E	−18,15	0

* After subtraction of control values, which averaged 415 counts/min./g.

In vitro determinations were done in duplicate, and both results recorded.

the sequential incubation method. Fifty mg aliquots of each HIFC preparation were used. *Sequential* rather than *simultaneous* incubation was chosen for the assay procedure for reasons to be discussed. Table III also gives the *in vivo* abilities of the same 7 preparations to enhance radioactive-cobalt-labeled B_{12} absorption from the gastrointestinal tract of pernicious anemia patients, measured by a modification(9) of the Schilling urinary excretion test(10). "*In vivo* units of potency" are the calculated relative potencies of the various HIFC preparations, with the frequently studied(9,11,12,13) Lederle fraction #186-1 arbitrarily assigned a value of one unit. It is evident that the 2 methods of HIFC assay show good agreement as to relative activity.

Table IV shows the effect of one ml aliquots of gastric juice from 4 patients with histamine-fast achlorhydria and Vit. B_{12} deficiency disease. As would be expected if the *sequential* incubation method were an assay for intrinsic factor, the gastric juice from the 2 pernicious anemia patients had no effect on Co^{60}-B_{12} uptake by rat liver slices, but the gastric juice from the patients with nutritional B_{12} deficiency and malabsorption syndrome markedly enhanced Co^{60}-B_{12} uptake.

Table IV also demonstrates that 1 mg aliquots of heparin and chondroitin sulfate have no effect on Co^{60}-B_{12} uptake by rat liver slices in the *sequential* incubation method. Since both these agents bind B_{12}(14), clearly the *sequential* incubation method is not a measure only of B_{12} binding by intrinsic factor.

Discussion. The fact that there was an optimal concentration of HIFC producing maximal Co^{60}-B_{12} uptake by rat liver slices suggested a working hypothesis that receptors for intrinsic factor exist on the rat liver slice, and that in the presence of calcium these receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached. This hypothesis was supported by the observation that liver slices incubated in a high concentration of HIFC, washed, and subsequently incubated in Co^{60}-B_{12} would take up much more Co^{60}-B_{12} than slices incubated in these agents together. Two other possibilities suggest themselves to explain the experimental results. They are that an inhibitor of intrinsic factor action may be present in HIFC, or that non-intrinsic factor substances may be present in HIFC which compete with intrinsic factor for available Co^{60}-B_{12}. Any of these 3 possibilities can prevent enhancement of liver slice Co^{60}-B_{12} uptake in the presence of large amounts of HIFC. It was for this reason that *sequential* incubation in HIFC and then in Co^{60}-B_{12} was selected as the assay procedure.

No *in vitro* test can be proved to assay intrinsic factor at the present time, since intrinsic factor has not as yet been isolated in pure form. The *sequential* incubation system has accurately reflected the *in vivo* potency of the HIFC preparations tested; it has accurately predicted the diagnosis of B_{12} deficiency due to lack of intrinsic factor (Addisonian pernicious anemia), when the differential diagnosis rested between Addisonian pernicious anemia and B_{12} deficiency on a basis other

TABLE IV. Sequential Incubation Assay of Gastric Juice from Patients with Vit. B_{12} Deficiency Diseases. Assay of heparin and chondroitin sulfate.

Assayed material	Liver slice uptake (counts/min./g) Controls	Assayed material
Gastric juice from patient with:		
Pernicious anemia	387, 342	397, 419
Idem	" "	370, 328
Nutritional B_{12} lack	298, 364	776
Malabsorption	404, 354	855, 859
Heparin	" "	411, 436
Chondroitin sulfate	" "	377, 376

All assays in duplicate; both results recorded.

than lack of intrinsic factor; and it has been demonstrated not to be a measure of B_{12} binding alone. These results would suggest that this system may be indeed an *in vitro* assay for intrinsic factor.

Summary. When rat liver slices are incubated in hog intrinsic factor concentrate (HIFC) and Co^{60}-B_{12} simultaneously, there is an optimal concentration of HIFC producing maximal Co^{60}-B_{12} uptake by the liver slices. Any deviation from this optimal level results in a decreased enhancement of Co^{60}-B_{12} uptake. Slices incubated in high concentration of HIFC, washed, and then incubated in Co^{60}-B_{12} take up much more Co^{60}-B_{12} than slices incubated in these materials together. From these facts the hypothesis was derived that receptors for intrinsic factor may exist on rat liver slices, and that these receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached. A possible *in vitro* assay for intrinsic factor is presented, based on sequential incubation of rat liver slices in intrinsic factor-containing buffer and then in Co^{60}-B_{12}.

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Effect of Chromatotrophic Hormone on Pigments of Anuran Skin.* (23841)

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Recently, it has been demonstrated that both guanophores and melanophores of anurans are under the influence of the chromatotrophic hormone, CTH(1). Furthermore the relative amount of guanine contained in the guanophore appeared to be governed by CTH. These results were questionable, however, in the light of Gunder's report(2) which stated that the term guanophore was a misnomer and that this chromatophore actually contains no guanine. In an attempt to clarify this problem, the present investigation was undertaken to determine whether guanine is indeed the pigment-like substance of the guanophore and furthermore, whether CTH restricts the amount of this purine in these cells.

Materials and methods. The present experiments were performed on normal and hypophysioprivic larvae of *Rana pipiens* and *Rana sylvatica*. Eggs of *Rana pipiens* were obtained by induced ovulation and those of *Rana sylvatica* were collected near Rochester, N. Y. A few adult *Rana pipiens* were also used. The hypophyseal placode was removed from embryos at an early tailbud stage and about 30 hypophysioprivic and partially hypophysioprivic larvae were selected for the ex-

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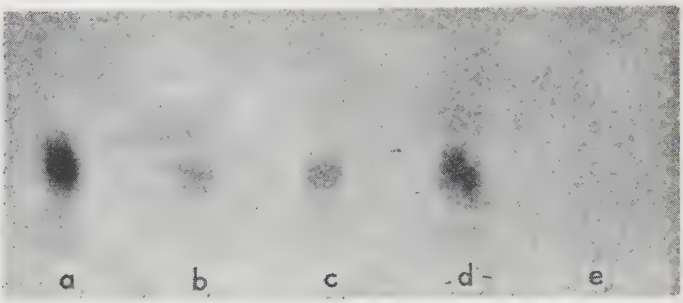

FIG. 1. Chromatogram illustrating guanine in dorsal skin extracts of *Rana sylvatica* larvae, mg guanine/g of dry skin. a. Control guanine; b. normal larva, 10 mg/g; c. partially hypophysioprivic larvae, 13.3 mg/g; d. hypophysioprivic larvae, 24.8 mg/g; e. hypophysioprivic larvae inj. with CTH, 2.3 mg/g, guanine present in such small quantities that it is not visible on the chromatogram.

periments. Into some of the larvae a CTH preparation (MSH Armour D216-155C) was injected intraperitoneally. Injections of .05 cc (10 μg) per tadpole were performed every other day so that each tadpole received a total of 3 injections. To eliminate contaminating effects of other hormones and for the purpose of potentiation, the hormone solution was hydrolyzed mildly by heating for 5 minutes in .1N NaOH at 100°C(1). When the larvae reached a length of 4 or 5 cm, the skin from the dorsal surface was peeled off with a watchmaker's forceps and was dried and weighed. In order to obtain enough material for each set of determinations, the skins of from 3 to 5 larvae were pooled. Guanine was extracted from both the larval and the adult skins by the method of Sumner(3). The various extracts were then studied by means of ascending paper chromatography. Most of the experiments employed Whatman #1 paper and a solvent of butanol, 4:acetic acid, 1: water, 1; however, several other solvent systems were also used. The temperature varied from 22-28°C during the course of the determinations, but was kept fairly constant during individual runs which ranged from 1 to 10 hours. The majority of runs were carried out for 1½ hours. In order to locate the guanine on the dried papers, a diazotization reaction was employed(4). As a result of this treatment, guanine appeared as an orange to purple spot. Guanine was also located by illuminating the dried papers with a U.V. lamp. Under such illumination a series of fluorescent spots also appeared which seem to be the pterins previously noted by Hama (5). In an attempt to quantitatively estimate the amount of guanine present in the various extracts, a series of known concentrations of guanine were chromatographed and developed with the diazotization reaction. Each spot was read on a Strip Reading Densitometer at 525 μ and the values obtained were used as reference standards for the guanine spots of the skin extracts. The values given in Table I were determined with these standards.

Results. A. *Guanine.* The results of more than 30 chromatograms indicate that a substance extractable in fairly large amounts from the skin of tadpoles and adults of *Rana pipiens* and *Rana sylvatica* has the same R_F value as a control guanine preparation. This substance is undoubtedly guanine. Furthermore, as is shown in Fig. 1, the presence of guanine in the skin is inhibited by CTH in *Rana sylvatica*. Thus the skin of the hypophysioprivic tadpole contains about twice the amount of guanine per dry weight as that of the normal tadpole. Skin of the partially hypophysioprivic tadpole, while containing less guanine than the completely hypophysioprivic tadpole, contains more than the skin of the normal larva. Injection of potentiated CTH into hypophysioprivic larvae is so inhibitory to guanine deposition that the skin of such tadpoles contains only one-tenth the amount that is found in skins of uninjected hypophysioprivic tadpoles. In *Rana pipiens,* the inhibitory effect is less striking. Skin extracts of such hypophysioprivic larvae contain more guanine than do the skin extracts of normal larvae, but there is little difference between the hypophysioprivic and hypophysioprivic-

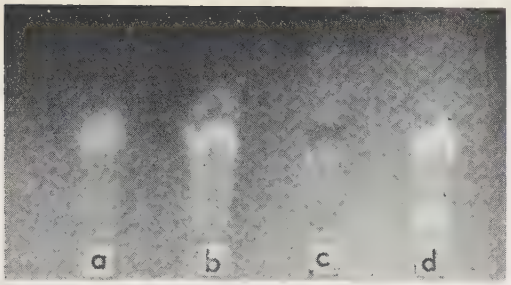

FIG. 2. Chromatogram showing fluorescing pterins in dorsal skin extracts of *Rana pipiens*. a. adult skin, b. normal larva, c. hypophysioprivic larva, d. hypophysioprivic larva inj. with CTH. The latter has returned to the pterin pattern typical for the normal larva.

CTH injected tadpoles in this regard.

B. *Pterins*. During the course of study, it was also possible to observe the effect of CTH on some of the pterins of anuran skin. The extraction methods employed were primarily designed for guanine; therefore, the fact that the pterins were observable was strictly fortuitous. As a result, these observations are of a general nature. As is shown in Fig. 2 and Table I there are obvious differences in the variety of pterins found in each of the experimental groups. The purple fluorescing substance is common to all groups. Unlike the skin of the normal larva, that of the hypophysioprivic tadpole lacks the yellow-green, the blue, and the green substances while in the skins of the hypophysioprivic-CTH injected larvae, the missing substances are restored.

Discussion. It seems probable that most of the guanine is located in the guanophore since the relative amount of this substance in the various experimental groups coincides perfectly with the histological picture reported earlier(1). Normal larvae possessing contracted guanophores all have a low guanine level while hypophysioprivic larvae with greatly expanded guanophores contain a high guanine content. As would be expected, partially hypophysioprivic larvae with slightly expanded guanophores are intermediate in their guanine level. The most striking correlation involves the hypophysioprivic-CTH injected larvae of *Rana sylvatica*. In these tadpoles a few days after injections of hormone, the guanophores of the dorsal surface seem to lose their pigment and practically disappear. Correspondingly, chromatographs of skin extracts of such tadpoles indicate a very large reduction in guanine content.

TABLE I. Average R_F Values for Various Fluorescent Substances in Skin Extracts of *Rana pipiens*.

Fluorescence of spot	Adult frog	Normal larva	Hypophysioprivic larva	Hypophysioprivic inj. larva
Yellow-green	.06	.07		.08
Blue, a	.06			
Purple	.20	.19	.21	.19
Blue, b	.27	.25		.25
Green		.34		.32
Dark, a			.04	
" b		.13	.16	.15
"		.27	.28	.28

The presence of guanine in the guanophore is in agreement with the classical observations of Ewald and Krukenberg(6) and Schmidt (7). Since 1954, Gunder has reported finding guanine in the skin of anurans when using chromatographic technics(8). She attributes her early lack of success to the technic employed (personal communication).

The observation that CTH plays a role in the formation of pterins and the deposition of guanine is interesting because it further extends the known activity of this hormone.

Summary. Chromatographic analyses of anuran skin extracts indicate that guanine is indeed the pigment of the guanophore. The guanine level is controlled by the chromatotrophic hormone, CTH. This hormone also stimulates the formation of the fluorescent pterins of the skin.

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Synergistic Action of Russell Viper Venom and Tissue Thromboplastin Extracts.* (23842)

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In 1945 it was noted that when an extract of acetone-dehydrated rabbit brain was mixed with Russell viper venom, a marked synergistic effect was produced as measured by the one-stage prothrombin time(1). Gollub, Kaplan and Meranze(2,3) have reported a similar synergistic action when rabbit lung and brain extracts were combined. A further study on the effect of mixing various tissue extracts and Russell viper venom on the prothrombin time seemed warranted.

Materials and methods. The rabbit brain was extracted with acetone and the extract prepared as previously described(4). The other tissues studied were thoroughly perfused with tap water and homogenized in a Waring blender with 3 parts physiological saline to 1 part tissue. Russell viper venom (Stypven) was supplied by the Burroughs-Wellcome Co. Labile factor (factor V) deficient human plasma was obtained by storing normal oxalated plasma at 4°C until the prothrombin time became 30 seconds or longer. Stable factor (factor VII) deficient bovine plasma was prepared by passing it through a Seitz filter according to the directions of Koller *et al.*(5). The prothrombin time was carried out by the technic of Quick.

Results. Prothrombin time of fresh human plasma using acetone-dehydrated rabbit brain as the thromboplastin reagent is 12 seconds. It has not been possible to find a tissue extract which yields a significantly shorter value, but curiously it is possible by mixing various tissues such as brain and placenta, and lung and placenta (Table I) to obtain a prothrombin time shorter than with either constituent alone. The synergistic action between Russell viper venom and various tissue extracts is more pronounced than between various tissues. Particularly striking is the short prothrombin time of 6 seconds obtained on human plasma with a mixture of rabbit brain and Russell viper venom.

In the preceding experiments normal fresh plasma was used. On diminishing the labile factor by storage, the prothrombin time with rabbit brain is prolonged. Thus, the value recorded in Table II is 32.5 seconds. With Russell viper venom alone, the prothrombin time is exceedingly prolonged and with the combination of brain extract and venom, no synergistic action is observed. When stable factor, or factor VII, is removed by filtration through a Seitz filter, a synergistic action with brain and venom is still obtained although the prothrombin time is only reduced to 19.5. It is likely that some prothrombin is removed by filtering plasma through a Seitz filter. A plasma from a patient with congenital stable factor deficiency with a prothrombin time of 27.5 seconds with rabbit brain extract, and 15.5 seconds with venom gave 6 seconds with the 2 reagents combined, the same as is obtained on normal plasma. In marked contrast, plasma from a case of congenital true hypoprothrombinemia showed little synergistic response to the combined rabbit brain-venom thromboplastin reagent. On the other hand, plasma from patients receiving Dicumarol showed a significant response to the combination of brain and venom reagent. An average value of 8.5

TABLE I. Synergistic Action between Tissue Thromboplastins and Russell Viper Venom as Measured by One-Stage Prothrombin Time on Fresh Human Plasma.

	Prothrombin time (sec.)				
	With reagent alone	With mixtures*			
		Venom	Placenta	Brain	Lung
Venom†	23				
Placenta‡	11½	11			
Brain	12	6	8½		
Lung‡	13	8	10	12	
Liver‡	46	8½	15	16½	15

* Equal volumes were mixed.
† 0.01% solution.
‡ Human placenta was used; lung and liver were obtained from freshly killed rabbits.

* This work was supported by a grant from the Nat. Heart Inst., N.I.H., U.S.P.H.S.

TABLE II. Synergistic Action between Rabbit Brain and Russell Viper Venom Tested on Plasma in Which Labile Factor and Stable Factor Were Experimentally Removed.

Plasma	Rabbit brain	Russell viper venom	Rabbit brain + venom*
	Sec.		
Normal	12	17	6
Labile factor exp. depleted	32.5	∞	31.5
Stable " " "	230	75	19.5
Congenital stable factor deficiency	27.5	15.5	6.0
Congenital hypoprothrombinemia vera	20.5	65.0	16.5
After Dicumarol:			
Subject 1	35	65.0	8.5
2	33	58	8.5
3	37	50.5	9.5
4	46	35	8.5
5	36	36.5	8.5

* A 0.1% solution of Russell viper venom was mixed with an equal vol of rabbit brain extract.

seconds was obtained.

Discussion. Tissue extracts probably contain several agents which have thromboplastin activity and the potentiation resulting from mixing various tissue extracts may, perhaps, be due to a "reshuffling of compounds in the thromboplastin complex or a rebalancing of functionally different thromboplastins," as Gollub and his associates(2) have proposed. There is also the possibility that since the tissues such as lung and placenta contain at least traces of blood, plasma thromboplastin may be generated, thus giving an additive effect to the preformed thromboplastin present in the tissues. The synergistic effect of Russell viper venom on rabbit brain extract is difficult to explain.

From the results recorded in Table II, it is evident that the activity of Russell viper venom is greatly influenced by labile factor, and when the latter is markedly depleted, as in stored plasma, the synergistic action of rabbit brain and venom is absent. Likewise, in the depletion of prothrombin, as in congenital hypoprothrombinemia vera, the synergistic action of brain and venom is very slight. The activity of Russell viper venom as measured by the one-stage prothrombin time is markedly influenced by both labile factor and prothrombin as is also rabbit brain extract, and when the two are mixed, very little synergistic action is observed if either of these two basic clotting factors is diminished.

Stable factor (factor VII) is not needed for the activity of Russell viper venom. Thus, the prothrombin time using venom as the thromboplastin reagent is the same for plasma from a patient with marked congenital reduction of stable factor as for normal plasma; and when the mixture of venom and brain extract is used, a prothrombin time of 6 seconds is obtained for both plasmas. The results obtained on the plasma of patients receiving Dicumarol are particularly significant. With rabbit brain, prothrombin times varying from 33 to 46 seconds were obtained while with venom, the range was 35 to 58, but there was no close correlation of the two sets of results. When a mixture of brain and venom was used as the thromboplastin reagent, an average value of less than 9 seconds was obtained. This is in harmony with the conclusion of various investigators that Dicumarol reduces the stable factor to a much greater degree than it does prothrombin. Rabbit brain furnishes a thromboplastin reagent for the prothrombin time test which is much more sensitive than Russell viper venom for stable factor deficiency. The substitution of the latter for rabbit brain can be fraught with danger in the control of Dicumarol therapy, as Wilson(6) has pointed out.

Summary. As measured by one-stage prothrombin time, mixtures of various body tissues may have thromboplastin activity greater than that of either of the tissues in the mixture. A marked synergistic action as to thromboplastin activity occurs between body tissue extracts and Russell viper venom. This effect is absent if the plasma lacks either prothrombin or labile factor (factor V). The thromboplastin activity of Russell viper venom is not influenced by the lack of stable factor (factor VII) in contrast to the high sensitivity observed with rabbit brain thromboplastin.

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Further Studies on Urinary Aldosterone in Human Arterial Hypertension.* (23843)

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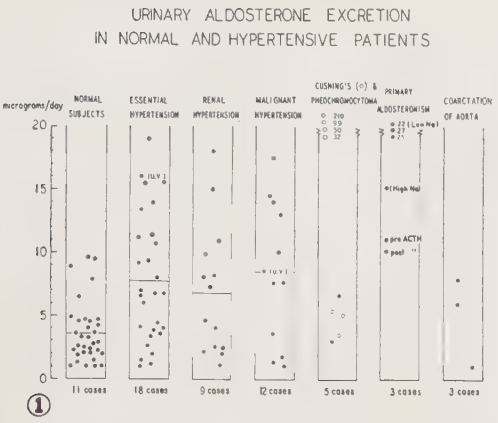
Two years ago, using a bioassay determination of a purified urinary aldosterone fraction, we reported a significant 2-fold increase in mean urinary aldosterone excretion of groups of severe essential and malignant hypertensive patients as compared to a group of normal subjects(1). Subsequent chromatographic study of this fraction revealed the possible occurrence of 6 other ultraviolet absorbing substances(2). This finding permitted elaboration of a new specific physico-chemical method for determination of aldosterone isolated from urinary extracts(3).

Because of extensive clinical and experimental evidence showing that adreno-cortical hormones with a predominantly mineralocorticoid activity may be directly concerned with the mechanism of arterial hypertension (4), we have applied this new chemical method to the study of 39 patients with essential, renal and malignant hypertension and another group of 11 patients with pheochromocytoma, Cushing's syndrome, primary aldosteronism, and coarctation of the aorta.

Method and subjects. The method used was previously reported(3). It is based essentially on isolation of aldosterone in a very high degree of purity and on its determination and identification by ultra-violet absorption at 239 mμ, reduction of blue tetrazolium and absorption spectra in 100% phosphoric acid and concentrated sulfuric acid. In the last 2 years we have had repeated difficulties with eluates from Whatman paper 1 and 2, whether it was specially selected for chromatography or not. Residues of blank eluates from these papers contain a number of interfering substances which react readily with a yellowish brown color after addition of tetramethylammonium hydroxide and blue tetrazolium(5). It has therefore been necessary to add a final purification procedure for separation of aldosterone from these interfering substances eluted from the Whatman paper.† A chromatographic glass column (internal diameter: 7 mm) is filled with Kieselgur (Hyflo-Super Cell, Johns Manville) 500 mg/500 mg water. The eluate residue of the aldosterone zone obtained in the last system (Bush B 5) is dissolved in 5 ml of benzene saturated with water. 15 ml of petroleum ether (D 0.67-0.69) are added and the mixture transferred to the column. The first eluate is discarded. Aldosterone is eluted by the second solvent fraction containing benzene (30 ml) and petroleum ether (10 ml). The Kieselgur and the solvents used are purified according to Hegedüs *et al.*(6) and Schindler *et al.*(7). Each patient was studied as follows: complete history with special emphasis on psychosomatic and emotional aspect of the patient's personality; detailed physical examination with special attention to the state of arteries, fundi and heart, electrocardiogram and teleoroentgenogram of heart, urine analysis with special attention to sediment, blood urea, phenolsulfonphtalein ex-

* This work was supported through grants of the Ministries of Health (Federal-Provincial Plan), the Life Insurance Medical Research Fund, New York, the Ciba Co., Montreal, and the Mead, Johnson Co., Canada.

† We found recently that these contaminants interfere little with the isonicotinic acid hydrazide reaction described recently by Weichselbaum and Margraf and which depends on the presence of a Δ^4-3 ketone group (8).



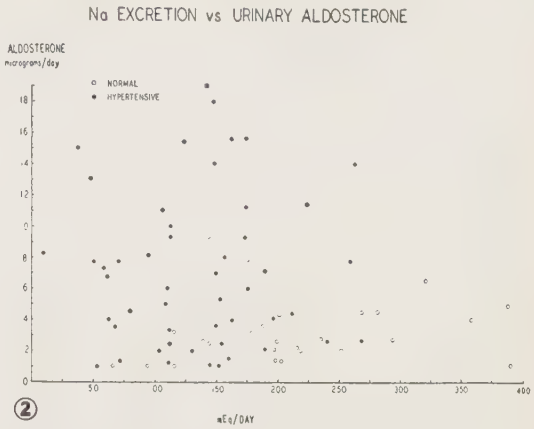


FIG. 1. Results of individual "spot" determinations done on normal subjects and patients with the various types of hypertensive cardiovascular disease.

FIG. 2. Urinary aldosterone excretion shows no correlation with urinary sodium in normal subjects or hypertensive patients on a self-selected diet containing 50 and 400 meq of sodium per day.

cretion, urea and endogenous creatinine clearance, serum potassium and intravenous pyelography. Retrograde pyelography, Rogitine and benzodioxane tests, and aortography were done when indicated. Patients were on ward diet without any restriction and all urine collections were done after a minimum of 48 and, in most cases, 72 hours of hospital admission. Exception was made for most patients with malignant hypertension who were on a low protein diet (30 g/day) and were not permitted to use the salt-shaker. Only one patient with essential hypertension was in congestive heart failure with generalized edema, and his aldosterone excretion was 3.3 μg/day. No other presented clinically detectable edema or venous distension. Venous pressure was taken in several patients and was within normal limits. In the clinical histories, 22 of the 39 patients complained of some degree of dyspnea on exertion. The presence of papilledema and of a diastolic pressure constantly above 130 mm of Hg were main criteria for differential diagnosis of the 12 patients with malignant hypertension. Nine of these were of renal origin and had very poor renal function with nitrogen retention and a high serum creatinine level. Hypertension of renal origin was diagnosed only when it was clear from the case history that the renal disease preceded the appearance of hypertension.

Results. Results are presented in 2 ways: (1) "spot" determinations done on normal subjects and patients with various types of hypertension (Fig. 1); (2) serial determinations done on one normal subject and 2 patients with early asymptomatic hypertension, one of benign essential variety and the other of renal origin secondary to repeated toxemia of pregnancy.

Thirty-three urinary aldosterone determinations in 11 normal subjects give a mean daily excretion of 3.6 μg/day, range 1 to 9.5 μg/day. Fifty-four aldosterone determinations were done in 39 patients with essential, renal

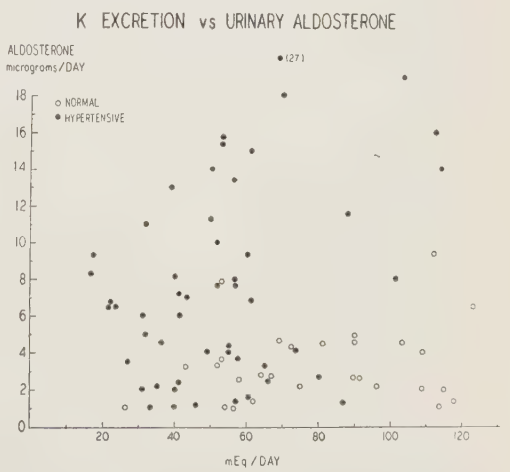


FIG. 3. Many patients with arterial hypertension excrete more aldosterone on a lower potassium intake than do normal subjects.

TABLE I. Aldosterone Excretion in Arterial Hypertension.

	Normal subjects	Hypertension		
		Essential	Renal	Malignant
No. of patients	11	18	9	12
No. of determinations	33	28	14	12
Mean (μg/24 hr)	3.6 $\pm$.42	7.8 $\pm$.96	6.9 $\pm$ 1.4	8.4 $\pm$ 1.4
"t"		4.1	2.99	4.22
"p"		<.001	<.001	<.001

and malignant hypertension. The mean excretion of each of these 3 groups was about twice the average normal excretion and about 55% of the patients showed an excretion higher than normal or in the higher range of normal. The significance of the differences between the means by the "t" test is such that the "p" value for the essential, renal and malignant groups is below 0.001 (Table I). These findings fully confirm our previous report(1a,b). Although these results show that in more than half of the patients with arterial hypertension, urinary aldosterone is in the high range or higher than normal, their significance is limited since they represent results of "spot" determinations. Therefore, one normal subject carrying his usual activities and on a self-selected diet was studied for 2 consecutive days in June 1956 and for 9 consecutive days in November 1956. His aldosterone excretion was quite constant between 1.3 to 6.5 μg/day despite marked fluctuations of urinary sodium from 66 to 386 meq/day. On the other hand, one patient with asymptomatic benign essential hypertension was studied for 5 consecutive days and her aldosterone excretion was 7, 15.7, 3.6, 9.3 and 14 μg/day with a fairly constant urinary sodium output between 150 and 170 meq/day.

A second patient with asymptomatic renal hypertension secondary to repeated toxemia of pregnancy showed similar variations of 12.1 (average of 2 days) 2.4, 4.5 and 7.2 μg/day. No reasons of acute stress, anxiety state, changes in sodium or potassium intake could be found to account for these variations in the 2 patients.‡

Two patients with proven pheochromocytoma had values within the normal range. Three patients with Cushing's syndrome showed variable results. One of these, who presented severe hypertension, hypernatremia (153 meq/liter), hypokalemia (2.45 meq/liter) due to bilateral adrenal hyperplasia, had a urinary aldosterone of 99, 50, 210, 32 μg/day(9). Because the critical state of patient's condition did not permit early bilateral adrenalectomy, he was given daily X-ray therapy over the pituitary region and testosterone propionate (25 mg 3 times a week). After 4 weeks of this treatment, the aldosterone excretion was 4.7 μg/day. A second patient with bilateral adrenal hyperplasia and with a normal blood pressure and normal sodium and potassium serum levels, had an aldosterone excretion of 5.3 μg/day which rose to 15.6 during intravenous ACTH infusion (25 units during 8 hours). The third patient with Cushing's syndrome due to a chromophobe adenoma of the pituitary had values of 3.5 and 5 μg/day. This patient was moderately hypertensive.

Three patients with primary aldosteronism were studied. One patient was studied by Dr. Arnold Relman at Mass. Memorial Hospital, Boston, under conditions of low and high sodium intake and before and during ACTH treatment. Results are indicated in Fig. 1. Three patients with coarctation of the aorta had values within the normal range. The pre-operative value on one such patient (followed by Dr. Nathan Sims, University of Vermont) was 7.8 and following surgical correction the values were 7.3 μg eight days post-operatively and 2.6 μg/day 26 days post-operatively.

Among the factors which could be related to aldosterone excretion in normal and hypertensive patients, the following were studied:

‡ Since submission of manuscript, a third patient with benign essential hypertension was studied for serial urinary aldosterone determinations and presented similar variations of 10.2, 1.2, 1.6, 15.5, 14.5, 4, 8.8 μg/day. This patient's only complaints were periods of dizziness, scotomas and nycturia of two months duration. No symptoms or signs of cardiac failure. Venous pressure: 70 mm H_2O. Endogenous creatinine clearance 100 ml/min. PSP excretion: 61% in 60 minutes.

urine volume, urinary sodium and potassium, urinary Na/K and K/Na ratios and cardio-thoracic ratio. No correlation could be observed between aldosterone excretion and sodium intake as reflected by urinary sodium which varied between 50 and 400 meq/day (Fig. 2). No correlation could be made with the Na/K or the K/Na ratios. Similar findings were also reported in normal subjects on unrestricted diets by Tait and his group(10).

No correlation could be made between aldosterone excretion and the urine volume, cardio-thoracic ratio or presence or absence of dyspnea on exertion. One finding of interest shows that many hypertensive patients have a higher aldosterone output on a lower potassium intake than the normal subjects (Fig. 3). This is in contrast with the findings of the stimulating effect of high potassium intake on the aldosterone excretion(3,11,12,13). These observations serve to emphasize that aldosterone excretion in hypertensive patients or in normal subjects on self-selected diets is the result of the interaction of many factors, several as yet unknown.

Conclusion. Our results indicate 1) increased aldosterone excretion in about 55% of patients, 2) a very significant difference between mean aldosterone excretion of patients with essential, renal or malignant hypertension as compared with normal subjects, 3) a much greater degree of fluctuation in serial daily aldosterone determinations in 2 early asymptomatic hypertensive patients as compared to a normal subject. These observations bring additional and direct evidence for an adrenal cortical disturbance in hypertensive cardio-vascular disease. Although the evidence is suggestive, it cannot be established at the present time if these findings play an etiological role in pathogenesis of arterial hypertension. They may provide the explanation for the successful use of salt depletion by chlorothiazide and mercurials as essential adjunct to the "effectiveness" of the present anti-hypertensive therapy.

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Propagation and Modification of Infectious Bovine Rhinotracheitis (IBR) Virus in Porcine Kidney Tissue Culture. (23844)

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The propagation of infectious bovine rhinotracheitis (IBR) virus(1,2) and its modification in bovine kidney tissue culture(3), as well as the adaptation of this virus to HeLa cells(4) have been previously reported. However, McKercher *et al.*(5) failed in attempts to isolate a cytopathogenic agent in porcine kidney tissue culture from nasal washings of cattle containing IBR virus. This report shows that IBR virus can be isolated and propagated serially in porcine kidney tissue culture (PKTC) with production of a typical cytopathogenic effect (CPE). Furthermore, such passages can result in a virus which does not produce any disease when inoculated intramuscularly or intranasally into susceptible cattle.

Material and methods. Virus. A 10% suspension of turbinate, larynx and trachea from 2 cows in acute phase of IBR infection was used to demonstrate primary isolation of IBR virus in PKTC. From part of the same respiratory tissues, IBR virus had been previously isolated in bovine kidney tissue culture (BKTC) and designated as Colorado I(2). The 7th passage of this virus in BKTC was also employed to initiate further passages in PKTC. *Tissue culture procedure.* The porcine kidney tissue cultures were prepared by trypsinizing kidney cortex of 1 to 8-week-old pigs, and suspending the cells in nutrient medium consisting of 0.5% lactalbumin hydrolysate and 5% horse serum in Earle's basic salt solution, to which 200 units of penicillin and 200 μg of streptomycin/ml was added. This mixture was dispensed in 0.5 ml amounts into culture tubes, and allowed to stand in a stationary position until good cellular growth was observed, following which the tubes were placed in roller drums. In the course of this work, it was noted that more extensive and rapid growth occurred when calf serum was used in the medium. Therefore, 10% calf serum which did not contain IBR antibodies was substituted for the horse serum in the growth medium but before use, the growth medium was replaced with the 5% horse serum medium. The propagation and maintenance of embryonic bovine kidney tissue culture cells has been described(2). *Neutralization tests.* Tests for IBR antibodies in the sera of cattle were performed in calf kidney tissue culture exclusively, as described previously(2,3), with IBR virus propagated in BKTC. *Cattle.* Cattle 4-8 months old, weighing 350-500 lb, regardless of sex or breed, were used for virulence and antigenicity studies. The susceptibility of these cattle to IBR was determined by absence of neutralizing antibodies in the serum. In addition, they were held in isolation for at least 2 weeks prior to inoculation, and daily temperature records as well as other observations were made to insure that the animals were normal before use. After inoculation, in addition to the use of serum-neutralization tests to demonstrate antibodies, some of the animals were challenged intranasally with 1-2 ml of virulent (low passage BKTC fluid) virus as described(3). No response was considered definite unless fever and one or more signs of illness such as increased respiration, anorexia, nasal discharge, hyperemia of the nasal mucosa, serofibrinous exudate covering the nasal mucosa, were observed.

Results. Propagation of virus. A 10% suspension of upper respiratory tissues from acutely ill cattle was centrifuged at 1,000 rpm for 10 minutes and 0.2 ml of the supernatant inoculated into each of several PKTC tubes. Two-three days after inoculation, a cytological change was observed, consisting of granulation and rounding up of the cells. This effect progressed until all cells became involved and finally fell off the wall of the tube. When 50-100% of the cells were affected, tissue culture fluid from the tubes was transferred in 0.2 ml amounts into each of several new tis-

sue culture tubes. A total of 10 serial passages in PKTC was made. The 10th passage material, referred to as O-10, was titered simultaneously in PKTC and BKTC, using the CPE as an indicator of end point. Ten-fold serial dilutions of the virus were prepared in tissue culture medium without serum, and 0.2 ml amounts inoculated into each of several tissue culture tubes for each dilution. The titer in PKTC was $10^{4.8}$ 50% tissue culture infectious doses ($TCID_{50}$), and $10^{5.3}$ $TCID_{50}$ in BKTC per 0.2 ml. This indicated that the virus had multiplied in the PKTC, since the end point of titration for the 10th passage would represent a dilution of at least $10^{-14.8}$ of the original tissue suspension.

IBR virus was also propagated in PKTC using as seed material virus that had been previously passed 7 times in BKTC. The tubes were inoculated with 0.2 ml of the infected tissue culture fluids, and all subsequent passages were made by transfers of the same amounts of infected fluid. Serial passages of the virus were performed in two ways: 1) transferring the tissue culture fluid when 90-100% of the cells showed a CPE; and 2) transfers at 1-2 day intervals or when approximately 25-50% of the cells were involved. In the first series after 60 passages, terminal dilution titrations were performed to select the virus particles that were present in the greatest number at that time. Ten-fold dilutions of the infected tissue culture fluids were prepared and at least 3 tissue culture tubes were inoculated with each dilution. The fluid from the culture inoculated with the highest dilution which produced a CPE was harvested, and the procedure repeated. Three such terminal dilution transfers were made. Several larger batches of virus were then prepared, using this third terminal dilution virus as seed material. This virus is henceforth referred to as P-60. Titrations of 2 batches of this virus material were performed simultaneously in porcine and bovine kidney tissue culture, with titers of $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ in PKTC, and of $10^{4.8}$ and $10^{6.3}$ in BKTC per 0.2 ml. In the series where transfers were made when only a part of the cells were involved, 100 serial passages were performed, followed by 3 terminal dilution steps. Again 2 batches of infected tissue culture fluid prepared from the third terminal dilution virus (RP-100) had titers of $10^{4.8}$ and $10^{5.8}$ $TCID_{50}$ per 0.2 ml in PKTC, and of $10^{5.8}$ and $10^{6.3}$ per 0.2 ml in BKTC. One lot titered only in BKTC had an end point of $10^{7.5}$. The cytological changes occurring with the use of P-60 as well as RP-100 in BKTC could not be differentiated from those caused by BKTC-propagated IBR virus.

Identification of the virus. Identification of O-10, P-60, and RP-100 virus passage material was performed with known IBR immune sera, utilizing neutralization tests in tissue culture. As controls either normal bovine sera or tissue culture media were employed. Equal quantities of undiluted immune sera or control fluids were mixed with concentrations of virus ranging from approximately 10-3200 $TCID_{50}$. These mixtures were incubated at 37°C for 2 hours and each serum-virus mixture inoculated in 0.2 ml amounts into each of 2-4 culture tubes, using either porcine or bovine kidney tissue culture, or both simultaneously. These cultures were observed regularly, with the final reading approximately 5-7 days after the beginning of the test. It was found that IBR immune sera neutralized each of the 3 virus lines as indicated by the absence of any cytological changes in the cultures while normal serum or tissue culture medium did not prevent the CPE.

Tests in cattle. Further evidence that the cytopathogenic agent propagated in PKTC is IBR was obtained by inoculation of 1-2 ml amounts of infected tissue culture fluid intramuscularly and intranasally in cattle, using each of the 3 virus lines (O-10, P-60, and RP-100). All virus lots were titered simultaneously in porcine and bovine kidney tissue culture prior to inoculation. Serum samples were taken from the animals at the time of inoculation and again 3 weeks later. None of the animals had IBR antibodies prior to inoculation. Some of the animals were challenged intranasally with known virulent virus at the time the second serum sample was taken. The results of these tests (Table I) show that none of the virus lines inoculated intramuscularly produced any significant evi-

TABLE I. Results of Intramuscular and Intranasal Inoculation of Cattle with Pig Kidney Tissue Culture Adapted IBR Virus.

Route of inoc.	Virus	$TCID_{50}$ inoc. (logs)	Clinical response: Fever	Clinical response: Other	Antibody response‖	Challenge response
Intramusc.	P-60	5.5	1/1*	0/1†	1: 3	0/1
	"	4.5	0/1	"	1: 7	"
	RP-100	6.5	"	"	1:19	"
	"	5.5	"	"	1: 3	"
	"	6.0	0/2	0/2	1: 7; 1: 9	NT§
	"	5.0	"	"	1:11; 1: 6	"
	"	4.0	"	"	1: 6; 1: 7	"
	"	3.0	"	"	1: 3; 1: 6	"
	O-10	6.3	0/1	0/1	1: 7	0/1
Intranasal	P-60	7.3	0/1	?‡	trace	0/1
	RP-100	7.3	"	0/1	0	1/1
	"	8.5	0/2	0/2	0; 0	NT
	O-10	6.5	2/2	2/2	1:19; 1:23	"

* Fever of 103.8 for one day. † Numerator = No. responding; denominator = No. tested. ‡ A slight hyperemia and nasal discharge noted for two days. § Not tested. ‖ Tested against 50-200 $TCID_{50}$ of virus.

dence of disease. All inoculated animals produced neutralizing antibodies, even when as little as 1,000 $TCID_{50}$ of RP-100 virus was used, and all those challenged proved to be immune. Following intranasal inoculation, O-10 produced a typical IBR and a good antibody titer. On the other hand, P-60 in one test caused only a slight clinical illness and a trace of antibodies, while RP-100 produced no disease or antibody response.

Discussion. As shown in results when either suspensions of IBR-infected respiratory tissue or low passage BKTC virus was inoculated into PKTC, a cytopathogenic agent was isolated. In one instance this agent was serially transferred for at least 100 passages. It may be noted in titrations of virus batches that the dilution end point was, in most instances, lower in PKTC than in BKTC tubes. This difference does not necessarily indicate a lower sensitivity of the virus for porcine tissue, but was probably due to the deterioration of the porcine kidney cultures before the virus reaction in the bovine cultures was complete, thereby preventing an accurate estimate of the end point with the higher dilutions of virus in PKTC. Evidence that this agent is IBR virus and not some other CPE-producing virus is shown by the fact that the effect is prevented by use of known immune sera, and that cattle inoculated intramuscularly developed antibodies which neutralized standard BKTC-propagated IBR virus. As shown in previous work(2,3), it is only by the intranasal route of inoculation, however, that all signs of illness observed in the field could be reproduced in experimental cattle. Thus, whether any change in the virulence of the virus occurred during passage in PKTC could be evaluated only by this route of inoculation. It appears that a definite change had taken place, because low passage virus (O-10) produced characteristic signs of IBR; P-60 only a questionable response; while as much as 320,000,000 $TCID_{50}$ of RP-100 produced no signs of illness. It is also of interest to note that in spite of this large amount of RP-100 virus inoculated, no antibody production could be demonstrated. The comparison between the results in cattle inoculated intranasally with high PKTC passage material (RP-100) and other animals inoculated with low PKTC material (O-10) demonstrates that passage in PKTC results in a virus population which lost much of its infectivity for the natural host. However, this phenomenon appears not to be dependent entirely on the porcine tissue itself, since it could also be observed to a lesser degree previously with BKTC passage virus. The same virus, Colorado I strain, after more than 40 passages in BKTC, did not produce any disease, and only 2 out of 4 animals developed neutralizing antibodies when inoculated intranasally with 200,000 $TCID_{50}$. On the other hand, 320 $TCID_{50}$ of low passage virulent virus did

cause typical signs of IBR and a definite antibody response(3). In spite of this decrease of infectivity, however, the antigenicity of the virus when inoculated intramuscularly, regardless of passage level or tissue culture employed, was not significantly changed as shown by serum neutralization titers of experimental cattle. Thus, the PKTC-propagated virus could be used as a vaccine with the additional advantage that such a product would be free of cattle viruses which might be present in bovine kidney.

Summary. 1. IBR virus was isolated in porcine kidney tissue culture from infected respiratory tissues of cattle, and produced a typical CPE on these cells. The virus was maintained for 10 passages and, when inoculated intranasally into susceptible cattle, produced signs of illness characteristic of IBR. 2. IBR virus passed previously through 7 passages in BKTC could be successfully propagated in PKTC in 2 separate attempts, and was passed 60 times (P-60) and 100 times (RP-100), respectively, in PKTC. 3. Inoculation of P-60 and RP-100 did not cause disease when inoculated either intranasally or intramuscularly into susceptible animals. Intramuscular inoculation of these viruses resulted in a specific antibody response.

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Conversion of Progesterone-4-C^{14} to Aldosterone by Perfused Calf Adrenals. (23845)

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Previous studies with isolated calf adrenals have demonstrated production of sodium retaining activity following perfusion with an artificial medium, both with(1) and without (2) added progesterone. The zone containing this activity migrated with cortisone in the toluene-propylene glycol(3) paper chromatographic system and with cortisol in the Bush C system(4), thus resembling aldosterone. Because of low yield of this bio-conversion, chemical identification of the aldosterone has not thus far been practicable. With isotopic technics, we have been able to show definitively that perfused calf adrenals can produce aldosterone from progesterone. Tritiated aldosterone was added to aldosterone-4-C^{14} isolated from a perfusate containing progesterone-4-C^{14}, and the mixture shown to have a constant C^{14} to H^3 ratio through 3 chromatographic systems before, and 2 after, acetylation to aldosterone diacetate.

Methods. Seven left calf adrenals were perfused for 2 hours with one liter of artificial perfusion medium(5) containing 101 μc/40 mg progesterone-4-C^{14}.† The progesterone was added to the medium in 5 ml of propylene glycol. The perfusion fluid was extracted 5 times with 500 ml of dichloromethane, and the wash fluid from the apparatus (800 ml) extracted 3 times with 400 ml dichloromethane. These extracts were dried over sodium sulfate, combined, and evaporated *in*

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† Progesterone-4-C^{14} was obtained from Tracerlab, Boston, Mass., (sp. act. of 1.71 mc/mM). The 20.5 mg obtained was diluted with 19.5 mg of non-radioactive progesterone.

TABLE I. Chromatographic and Isotopic Characterization of Aldosterone.

System No.	Composition		Ref.	Aldosterone mobility, cm / hr	Cortisol®	% counted	C^{14} cpm	H^3 cpm	Ratio, tritium /carbon
I	E_2B		7	24.5/20	.71	5	302	7954	26.3
II	Benzene	4	4, 8	43.0/15	1.4	10	387	9782	25.3
	Methanol	2							
	Water	1							
III	Bush C		4	16.7/ 5	1.0	15	416	10276	24.7
IV	Cyclohexane	4	8	27.4/15		20	441	11461	26.0
	Dioxane	4							
	Methanol	2							
	Water	1							
V	Cyclohexane	4	8	31.5/12		25	326	8674	26.6
	Benzene	2							
	Methanol	4							
	Water	1							

C^{14} counts at Tap 3, 10-25 V. H^3 counts at Tap 10, 10-∞ V. Bkgd: 2.9 cpm, Tap 3; 91 cpm, Tap 10.

vacuo. Isolation of aldosterone was performed on the dried extract. An aliquot was run in the toluene-propylene glycol system(3) for 96 hr, and the zone containing cortisone was eluted with ethanol. Cortisone was then separated from aldosterone by running in a toluene-ethyl acetate-methanol-water system(4) for 5 hours, in which aldosterone migrates in the cortisol zone. This zone was located by its ultra-violet absorption, and eluted. Approximately 0.5 μg of tritium labelled aldosterone of high specific activity‡ and 52 μg of authentic aldosterone§ were added to the radioactive carbon material from the Bush C system. Non-radioactive aldosterone was added to facilitate location of the aldosterone zones by ultra-violet absorption. The steroid mixture was chromatographed in 3 successive systems (Table I). Regions containing aldosterone were located each time by their ultra-violet absorption, with reference to that of cortisol and cortisone standards. Each paper chromatogram was scanned for carbon activity using an automatic paper chromatogram scanner (Eisenberg, F. unpublished) fitted with an 0.8 mg/cm^2 cellophane window. In each instance, carbon activity corresponded exactly with ultra-violet absorption. Regions containing aldosterone were eluted with ethanol, and an aliquot taken for double isotope assay, the remainder dried and used for the succeeding system. Following third chromatography of free steroids, the mixture was acetylated overnight to aldosterone diacetate with 0.1 ml acetic anhydride and 0.2 ml pyridine. Aldosterone-21-monoacetate (Ciba) was acetylated in the same manner and then run in parallel as a chromatographic standard. The diacetates were run in 2 different systems (Table I). Aldosterone diacetate migrates with Δ 4-androstene-11β ol, 3, 17-dione in System IV and with adrenosterone in System V and these steroids were accordingly run as standards. Scanning and isotope assays were carried out exactly as for free steroids. Tritium and carbon were counted in the Packard Tri-Carb Model 314-DC Liquid Scintillation Spectrometer. Toluene containing 0.3% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-di [2-(5-phenyloxazolyl)] benzene (POPOP) was utilized as counting medium. Carbon activity was obtained at photomultiplier tube voltage tap 3 (810 V.) using a 10-25 volt window, and carbon-plus-tritium activity was counted at tap 10 (1380 V.) using a 10-∞ volt window. The ratio of carbon counts at tap 10 to those at tap 3 was 2.78. Counting error was less than $\pm$ 1.5% in each instance.

‡ Tritiated aldosterone (sp. act. approx. 1.1 μc/μg) was kindly supplied by Drs. Ralph Peterson and Bernard Kliman of NIAMD, NIH, Bethesda, Md. It was obtained by tritiation of aldosterone-21-monoacetate (Ciba) by the Wilzbach(6) technic. Free aldosterone was recovered by enzymatic hydrolysis of the acetate and purified by several successive chromatographic separations.

§ Authentic non-radioactive aldosterone was obtained from Dr. H. L. Mason, Mayo Foundation.

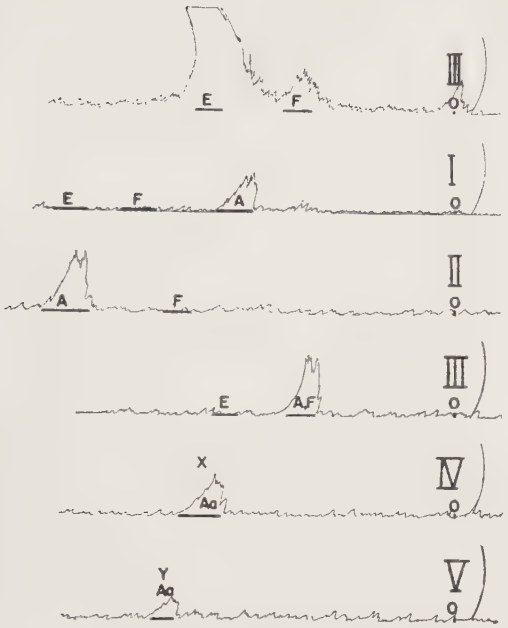

FIG. 1. Carbon radioactivity scan of paper chromatograms. Roman numerals refer to paper chromatographic systems listed in Table I. The top 2 chromatograms were recorded at 1500 cpm full scale deviation and the remaining 4 chromatograms at 500 cpm full scale. Horizontal bars indicate positions of standards. O, origin; E, cortisone; F, cortisol; A, aldosterone; Aa, aldosterone-di-acetate; X, Δ^4-androstene 11β ol, 3,17-dione; Y, adrenosterone.

Results. Fig. 1 (III, top curve) shows the C^{14} radioactivity scan of the Bush C chromatogram which followed initial separation of aldosterone from cortisol in the Zaffaroni system. To the material eluted from the cortisol zone of this Bush C chromatogram was added tritiated and non-radioactive aldosterone.

In each of the 5 chromatographic systems (Fig. 1, I-V) in which the mixed isotopes were run with authentic aldosterone, the ultraviolet absorption corresponded exactly with the zone containing C^{14} radioactivity as measured on the automatic scanner. Fig. 1 shows also location of standard run in parallel with aldosterone and its diacetate in each system: in all cases it was identical with that of radioactive aldosterone. Ultra-violet absorption was easily detected even after final chromatography in System V.

The tritium:carbon isotope ratio remained constant (Range 24.7-26.6) throughout the 3 chromatographic separations as free steroid and the 2 after acetylation, as shown in Table I. This constitutes excellent evidence for presence of radioactive aldosterone in the adrenal perfusate. It is concluded that radioactive progesterone was converted to radioactive aldosterone by the perfused calf adrenal.

No precise estimate of yield could be made. However, the activity of the purified radioactive aldosterone indicated about 0.1% conversion of progesterone-4-C^{14} to aldosterone. This can be compared with an estimated 0.5% yield based on sodium retaining activity of a calf adrenal perfusate(1).

Although there was no C^{14} activity in cortisol or cortisone zones of the E_2B (I) system, indicating complete separation of aldosterone from these steroids, a small amount of activity running slower than aldosterone was detected.

Summary. Calf adrenal glands were perfused with progesterone-4-C^{14} and the presence of radioactive aldosterone in the perfusate was established by isotopic and paper chromatographic technics.

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Ineffectiveness of Factor 3-Active Selenium Compounds in Resorption-Gestation Bioassay for Vit. E. (23846)

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Factor 3, described in 1951 as a new dietary agent(1), has recently been obtained in purified form.* It has been characterized as an organic substance containing selenium as an integral constituent of the molecule(2). The factor is very different from tocopherol: it is of low molecular weight, strictly water soluble, and stable against oxidation and acid hydrolysis(3). It was extremely effective in prevention of some fatal deficiencies, most of which have hitherto been attributed to lack of Vit. E. Evidently, these various diseases develop only if lack of both of these two essential factors coincides.† Thus far, it has been demonstrated that Factor 3 prevents liver necrosis in the rat; multiple necrotic degeneration (heart, liver, muscle, and kidney necrosis) in the mouse(4); exudative diathesis in the chick(5) and in the turkey(6); as well as dietary liver necrosis in the pig(7). Necrotic liver degeneration in the rat has been used as an assay system during the investigation of the factor. In preventing this fatal disease, 0.7 μg of selenium in form of α-Factor 3‡ per 100 g of diet afford 50% protection (8). Approximately 1,000 times as much Vit. E gives the same effect. It seemed important to investigate the influence of Factor 3 on fetal resorption in the pregnant rat since this classical symptom of Vit. E deficiency is the basis of the official bioassay procedure for determining Vit. E activity. The establishment of the International Standard and the International Unit of Vit. E is based upon it (9), as is the National Formulary X factor of 1.36 for relative potency of natural *vs.* synthetic α-tocopherols(10). Using the Mason-Harris modification(11) of Evans original resorption-gestation bioassay for Vit. E(12), and selenocystine and selenite as Factor 3-active supplements(8), we find that Factor 3-active compounds do not affect the Vit. E assay even when supplied in large excess.

Materials and methods. Two Factor 3-active compounds (selenocystine HCl and Na_2SeO_3) were bioassayed at levels supplying a total of 1, 10, or 100 μg of selenium (Table I). An additional level of 1 mg Se as H_2SeO_3 was also tested. The test substances were administered orally in aqueous solution by stomach tube, as well as intraperitoneally in doses of 0.2 ml on each of 5 consecutive days of pregnancy. The reference standard *d*-α-tocopheryl acetate, diluted in olive oil, was fed orally from a calibrated dropper in total doses of 0.2, 0.4, and 0.8 mg on the 4th through 8th days of pregnancy. Assay animals were female rats raised from weaning on diet low in Vit. E(11,13). When they attained a weight of 150 g they were mated with normal male rats, allocated into comparable groups, supplemented with either test or standard substances, and killed on the 20th day for examination of uterine contents. Only animals which had at least 4 implantation sites were used. Those which had at least 1 live fetus *in utero* were considered positive responses. Those with fetuses all dead or resorbed were negative responses. The proportion of positive to total responses in each group represents litter efficiency. The dosage which induces a 50% litter efficiency is known as the median fertility dose (MFD). The Factor 3 activity of selenocystine HCl and sodium selenite was determined, using the

* Schwarz, K., *et al.*, to be published. Since various compounds of selenium show greatly different biological activity, the term "Factor 3" is used to designate the biologically active, selenium-containing component, or components, present in nutrients and other materials of natural origin.

† The protective effect of L-cystine is caused by trace contamination with Factor 3-active selenium. (Schwarz, K., to be published).

‡ In acid hydrolysates of kidney powder, at least 2 chemically different but related components with Factor 3 activity have been detected. These have been designated α- and β-Factor 3. (Schwarz, K., Mason, L. H., and Foltz, C. M., to be published).

TABLE I. Vitamin E Bioassay of Factor 3-Active Selenium Compounds (8-10 Rats/Group).

Supplement	Mode of admin.	Total dose per animal	Amt of selenium per dose	Factor 3 (Fischer u)* per dose	Litter efficiency (%)	MFD (mg)
d-α-tocopheryl acetate	Oral	.2 mg			7	.39
		.4			50	
		.8			94	
D,L-selenocystine hydrochloride	Oral & I.P.	2.6 μg	1 μg	6.9	0	
		26	10	69	0	
		260	100	690	0	
Sodium selenite (Na_2SeO_3)	*Idem*	2.2	1	7.3	0	
		22	10	73	0	
		218	100	730	0	
Selenious acid (H_2SeO_3)	"	2.18 mg	1 mg	(5000)†	0	

* 1 Fischer unit is amount required daily/animal by Fischer strain of rats to achieve 50% protection against liver necrosis. For details concerning these tests see reference 8.

† Calculated from values obtained with SeO_2(8).

prophylactic rat assay against necrotic liver degeneration and the inbred Fischer 344 strain of rats. Values reported for Factor 3 were the result of series of assays at various dose levels(8). The pre-depletion method was used. Results are expressed in daily units for the Fischer animal, a unit constituting the amount required per day to afford 50% protection against liver necrosis.§

Results. In the resorption-gestation test, the calculated MFD for the Vit. E standard was 0.39 mg *d*-α-tocopheryl acetate (Table I). The results for Factor 3 preparations at all levels of supplementation were negative. Although the i.p.-administered Factor 3 preparations were completely absorbed, they exerted no tocopherol-like activity. The results show clearly that Factor 3-active selenium compounds do not influence the standard assay for Vit. E. However, these results do not imply that gestation is normal in the absence of Factor 3. Under conditions of the experiment, an ample amount of this agent is supplied by "vitamin-free" casein in basal diet, since this material is a good source of Factor 3(3).

The finding clearly supports the concept that Factor 3 and Vit. E do not replace each other but are independent, essential dietary agents. A possible explanation of the phenomenon that simultaneous lack of both agents is the cause of certain fatal deficiency diseases has been proposed. It is based on the assumption that Vit. E and Factor 3 participate independently in *alternate* pathways of oxidation-reduction, *i.e.*, electron transfer, in intermediary metabolism(14).

Summary. Factor 3-active selenium compounds, (selenocystine, sodium selenite and selenious acid) were bioassayed for Vit. E activity using standard rat resorption-gestation technic. These substances, administered in excess either orally or intraperitoneally, do not affect the bioassay of Vit. E even though they are highly effective against a variety of other deficiencies preventable by tocopherol, such as dietary necrotic liver degeneration and exudative diathesis.

§Factor 3 requirement of Fischer strain is 2.1 times as much as that of previously used Sprague-Dawley strain of rats, *i.e.*, 1 Fischer unit is equivalent to 2.1 Sprague-Dawley units.

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On the Mechanisms of Calcification.* (23847)

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Studies of the mechanism of calcification have been dominated by a single approach, that formulated by Robison(1): calcification involves some booster mechanism by which the product of the concentrations of calcium and phosphate is elevated locally so as to induce a precipitation of bone salts from saturated or slightly undersaturated tissue fluids. An important weakness in this approach is the necessary assumption that the tissue fluids are not already supersaturated with respect to bone salt. Actually, the older literature(2,3, 4) and recent results(5,6) have demonstrated rather conclusively that the serum and, most probably, the extracellular fluids are *supersaturated* with respect to the mineral phase of bone. More recently, in recognition of this supersaturated state of body fluids, attention has turned to the possibility that the mechanism of calcification may involve the crystal seeding or a surface-catalyzed induction of crystal formation. Indeed, quite some time ago(7), it was clearly shown that bone mineral would itself induce crystal formation from normal serum ultrafiltrates. These findings were extended and the ability to induce the formation of apatite crystals was shown also to reside in the collagen molecule, although specificity of collagen for such crystal induction was only crudely demonstrated(5, 8). More recently, however it has been claimed, on evidence from electronmicroscopy and X-ray diffraction technics, that only *one* crystallographic form of the collagen molecule is capable of inducing apatite crystal formation from supersaturated solutions of calcium and phosphate(9). Collectively, these studies make the *principle* of catalytic crystal seeding an attractive hypothesis at present. However, several questions arise: Do all collagens catalyze equally well? All connective tissues contain collagen, why don't all connective tissues calcify? Is the same mechanism, crystal seeding, operative in both osteoid *and* cartilage? As stressed recently(10), there are differences between calcification in osteoid and in the provisional zone of calcification in cartilage.

To obtain partial answers to some of these questions, an attempt was made to prepare purified, reconstituted(11) collagen fibers from a number of different tissues. It was hoped that these various collagen preparations could be compared quantitatively for their ability to induce crystal formation from supersaturated solutions of calcium and phosphate. Good yields of relatively pure, reconstituted fibers were obtained from skin and tendon. However, the usual reagents(11) employed in dissolving collagen from other tissues failed to dissolve collagen from bone sections previously demineralized with ethylene diamine tetra acetic acid (EDTA) at pH 7.4. Whether the EDTA-treated bone had been altered by the demineralizing procedure

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or whether the collagen native to bone has properties different from connective tissue collagen is a question not yet fully answered. Similar EDTA-treatment did not adversely affect the yield of reconstituted collagen fibers from skin. In any event, having failed to prepare collagen from bone, the tissue of most interest, a compromise was effected. Sections of skin, tendon and bone were merely demineralized with EDTA and the crude tissue residues tested directly for their ability to induce crystal formation from solutions of varying calcium and phosphate content. While it is not possible to attribute the seeding results obtained to the collagen molecule *per se*, there were no marked differences in the ability of the three tissues to seed crystal formation. Bone matrix seemed to "take up" calcium ions even when crystals did not form.

Methods. Preparation and treatment of tissues. Fresh calf legs and tails were obtained at the time the animals were sacrificed. The epiphyseal portion of the tibia was cleaned free from non-osseous tissue and sawed into blocks of approximately 1 cm^3. The skin and tendons were dissected from the tails and cut into similar small pieces. Samples of 1 g of each tissue were suspended in 300 cc of saturated EDTA (Versene) solutions at pH 7.4 for a period of 12 days, the solutions being changed on the sixth and ninth day to insure complete removal of mineral. After such treatment, bone was found by spectrographic analysis† to contain less than 1 mg of calcium per 100 g of tissue. The tissues were washed free of Versene twice with 500 cc aliquots of normal KCl solution and once with distilled water. All tissues were stored at subzero temperatures until used in the seeding experiments. *Preparation of solutions.* The calcium and phosphate solutions were prepared with CO_2-free distilled water, buffered at pH 7.4 with 0.02 *M* Veronal, and made up to physiologic ionic strength with KCl. The potassium chloride solution was similarly prepared. To inhibit bacterial growth 5 cc of chloroform per liter of solution were added, and the flasks were sealed with paraffin and parafilm(5,12). *Procedure.* Approximately 1 g of "decalcified" tissue was added into 100 cc of solutions of either constant calcium and variable phosphate concentrations or *vice versa*. The flasks were then sealed and equilibrated for 10 days at 37°C with gentle shaking. At the end of this period, the pH of the solutions was recorded and the fluid filtered through molecular filters. The filtrate was then analyzed for calcium by the method of Sobel and Hanok(13) and for phosphorus by the method of Fiske and SubbaRow(14). The calcium and phosphate removed from the original solution by the tissue served as a criterion of its calcification.

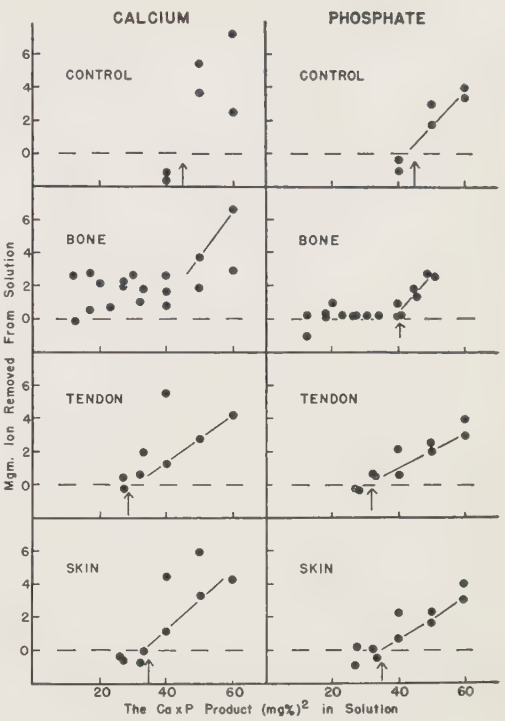

FIG. 1. Induction of crystal formation in the calcium:phosphate:water system by collagenous residues of skin, tendon, and bone. In these graphs, the product Ca × P is plotted *vs* the amount of calcium and phosphate (P) removed from solution by the organic phase. Relative scatter of points is not a true measure of the reproducibility since the solutions varied widely in the Ca/P ratio. Note that, in bone, calcium was removed from solution unaccompanied by phosphate.

Results. The results of these experiments have been summarized in Fig. 1. The degree of saturation of the solutions has been expressed with simple calcium and phosphate product, where calcium and phosphorus val-

† Courtesy Dr. L. Steadman, University of Rochester Atomic Energy Project, Rochester, N. Y.

ues were expressed as mg %. Based on the ionic strength and pH, this simple ratio can be converted to the thermodynamic product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$, by dividing by 18.6(5,12,15). In normal human sera, these products vary from about 18 to 35(16), while the cow normally has a serum product equivalent to about 35(17). In expressing serum products, one must correct for protein-bound calcium; thus a serum containing 10 mg% Ca and 3 mg % P would have an *ion* product of about 18 (6x3(16)).

As previously shown, solutions of calcium and phosphate, in the absence of solid phase, are stable and do not precipitate until the K_{sp} of $CaHPO_4 \cdot 2H_2O$ is exceeded(5). This stability is somewhat surprising since such solutions are frequently very supersaturated with respect to hydroxy apatite, the only stable phase at physiological pH(5). In these experiments, the K_{sp} of $CaHPO_4 \cdot 2H_2O$ corresponds to a Ca x P product of about 45. In confirmation of the earlier results, control experiments (no solid phase) were stable at a Ca x P product of 40 but precipitation occurred when the product was 50 or greater.

Also, in confirmation of earlier studies(4), the addition of collagen induced crystal formation at products below the point of spontaneous precipitation, in the range of 30 to 35. Surprisingly, perhaps, skin and tendon appeared to be at least as efficient in providing nucleation centers as did bone. The one unique property exhibited by bone matrix was its ability to bind calcium. This observation though reproducible and unequivocal may be in part artifactual in origin. Acid mucopolysaccharides have been demonstrated to possess cation-binding properties(18,19). Such substances may have been removed from the skin and tendon preparations by the prolonged EDTA-treatment and frequent washings in KCl buffer. Even so, this represents a major and unique property of bone matrix. It contains cation-binding substances even after prolonged washing and extractions. In these experiments, the binding of calcium by bone matrix renders it difficult to determine precisely the product (Ca x P) at which crystallization first occurred. As plotted in Fig. 1, the value represents the initial product, uncorrected for calcium removed from solution.

We have, however, other results obtained with a slightly modified technic. In these cases, the demineralized bone sections were placed in the solutions as before, but the solutions were decanted and *replaced* several times during the first 24 hours. Under these conditions (where calcium-binding could not materially lower the Ca x P product of the *final* mineralizing solution), the induction of crystal-formation occurred at products in the range of 30 to 35. Similar results were obtained with rat bone (unpublished). Human bone specimens, too, failed to mineralize at products below 30 (unpublished).

These results, in themselves, offer further evidence that the demineralization by EDTA was essentially complete. Had seed crystals remained behind in the tissues, mineralization would have been observed at even the low Ca x P products(5).

These results, then, raise more questions than they answer. How does human bone matrix become mineralized when the normal adult product, Ca x P, in the extracellular fluids is around 18? Presumably, some sort of "booster mechanism" will be required. Though a number of suggested "booster mechanisms" have been made in the literature(1, 20,21,22), none has gained universal acceptance. In the rat and the cow, with normal products of 35, why do not all collagenous tissues calcify? In these species, at least, some inhibitory mechanism must operate to prevent mineralization of extraskeletal collagen fibers. To our knowledge, only one such inhibitory mechanism has been postulated(23).

Clearly, the concept of a simple collagen-induced epitaxy of bone mineral is not adequate to account for the site-specific mineralization seen in the normal animal. Enzymes and cells will, in all probability, be credited with localized functions(24), though their role still remains unclear.

Summary. Specimens of skin, tendon, and cortical bone from the calf were exhaustively demineralized with EDTA at pH 7.4. These tissues were then equilibrated with solutions of varying calcium and phosphorus content. All 3 tissues, presumably because of their collagen content, were able to induce formation of

hydroxy apatite crystals from otherwise stable solutions. In contrast to skin and tendon, bone removed calcium from all solutions, but all three tissues were equally efficient in crystal seeding.

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Effect of Heparin on Serum Lipids Following Intravenous Administration of Fat Emulsion in Dogs.* (23848)

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The evidence that intravenous administration of heparin is capable of clearing alimentary lipemia *in vivo*(1,2,3), and that post-heparin plasma decreases turbidity of lipemic plasma(3,4) and of synthetic fat emulsion(5, 6) *in vitro* is well documented. Bragdon and Havel(7) and French and associates(8) have shown that administration of heparin increased rate of removal of intravenously injected lipoproteins and fatty chyle respectively. Meng and Youmans(9) and Grossman and Strub(10) found that heparin injected simultaneously with synthetic fat emulsion also accelerates the disappearance of the infused fat from the blood circulation. The present work was designed to study: (1) changes in serum lipids following intravenous administration of an olive oil emulsion, and (2) effect of heparin on removal of the infused fat and other serum lipids.

Methods. Healthy adult male dogs weighing from 9 to 15 kg were used. All animals were fasted for 24-36 hours prior to use and experiments were carried out under sodium pentobarbital (Nembutal Sodium) anesthesia. One gram of fat per kilo was infused intravenously as a 10% olive oil emulsion which was stabilized with purified soybean phos-

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phatide,† 0.5%; Demal-14,† 0.5%, Span 20,† 0.25%; sodium cholate, 0.1% and dextrose, 5%. The infusion was given by drip and completed in 30 minutes. Heparin sodium,‡ 0.5 mg/kg was injected intravenously 5 minutes prior to administration of fat emulsion; an additional amount (1 mg/kg) was mixed with the emulsion and was given by intravenous drip. Eight dogs received fat emulsion + heparin and 10 dogs received only the emulsion. Blood samples for serum lipids, including total fatty acids, total cholesterol and lipid phosphorus were drawn before, and at intervals during and after infusion of fat. Serum lipids were determined as follows: Total fatty acids by the method of Smith and Kik(11), total cholesterol according to Hoffman(12), and the lipid phosphorus according to Youngberg and Youngberg(13). The hematocrit value was obtained by centrifuging Wintrobe tubes which contained oxalated blood for 30 minutes at a rate of 2,500 rpm.

Results. Fig. 1 shows the changes in serum lipids following intravenous administration of olive oil emulsion in both heparinized and non-heparinized dogs. The results are expressed in terms of differences from initial levels. The vertical lines through each point on each curve represent the magnitude of the standard error of the mean. It can be noted that the serum total fatty acids were increased in both groups of animals during the 30-minute infusion period. However, the serum total fatty acids returned to the pre-infusion level within one and one-half hours after fat in the heparin-treated group ($p = 0.280$), while it remained significantly at a high level even 3 hours after completion of fat infusion in the animals receiving no heparin ($p = 0.037$).

A very gradual and progressive increase in serum total cholesterol was observed in dogs receiving no heparin (Fig. 1). The increase was significantly higher 2 and 3 hours after completion of fat infusion than that of the preinfusion level ($p = 0.050$ and 0.004 respectively). Contrary to that of the non-heparinized animals the serum total cholesterol was consistently decreased in the heparinized dogs (Fig. 1). The decrease was statistically significant at 15 and 30 minutes during ($p = 0.006$ and 0.005 respectively) and 30 and 60 minutes after infusion ($p = 0.058$ and 0.015 respectively). The differences in average serum total cholesterol between the heparin- and non-heparin-treated animals are highly significant at all points ($p =$ from 0.001 to 0.026).

The changes in serum lipid phosphorus following intravenous administration of fat emulsion were not consistent in either the heparin-treated or non-heparin-treated animals (Fig. 1).

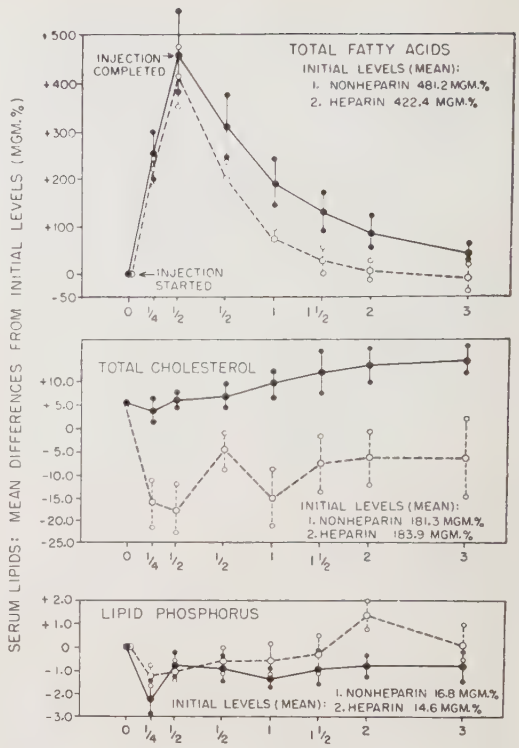

FIG. 1. Changes in serum lipids: Mean differences from initial levels. ●——●, non-heparinized dogs (10 dogs); ○---○, heparinized dogs (8 dogs). Vertical lines represent stand. error of mean.

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‡ Heparin sodium was kindly supplied by Dr. L. L. Coleman, Upjohn Co., Kalamazoo, Mich.

Discussion. It is well established that the clearing of lipemic plasma or of synthetic triglyceride emulsion by post-heparin plasma *in vitro* is lipolytic in nature. Clearing of alimentary lipemia *in vivo* following intravenous administration of heparin is most likely of the same mechanism, namely hydrolysis of the triglyceride component of plasma chylomicrons and lipoproteins. As demonstrated by Meng, *et al.*(14) synthetic triglyceride emulsion particles, as were used in the present study, are probably readily "coated" by plasma proteins forming chylomicrons and lipoproteins similar to those of alimentary lipemia. Intravenous injection of heparin in some way produces clearing enzyme which hydrolyzes the triglycerides releasing unesterified fatty acids. As reported by Havel and Fredrickson(15) the unesterified fatty acids can leave the blood stream very rapidly. It is not surprising that intravenous injection of heparin increased the rate of removal of the intravenously infused fat as was observed in the present investigation.

Lever and Waddell(16) found that intravenous infusion of cottonseed oil emulsion into patients with hypercholesteremia lowered the serum cholesterol. However, results of our study showed a gradual and progressive increase in serum total cholesterol. This may be due to (1) the differences of oils used (cottonseed *vs.* olive oil) and (2) the dogs used in the present work were not hypercholesteremic.

We are unable to explain why heparin was capable of lowering serum cholesterol in the dogs receiving fat emulsion. It is known that cholesterol esters are not hydrolyzed by the lipemia clearing enzyme. However, Basu and Stewart(17), Constantinides and coworkers(18), and Friedman and Byers(19), have found that heparin decreases serum cholesterol levels in hypercholesteremic patients, cholesterol-fed rabbits and Triton WR-1339 (oxyethylated tertiary-Octyphenol-formaldehyde) injected rats respectively. According to Basu and Stewart(17) the decrease occurs in both free and esterified cholesterol.

Summary. Intravenous infusion of olive oil emulsion produced elevation of serum total fatty acids and possibly total cholesterol, but without consistent changes in lipid phosphorus. Heparin injection simultaneously with fat infusion increased the rate of removal of the intravenously infused fat and lowered serum cholesterol, but produced no significant changes in lipid phosphorus.

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Differentiation of Yeasts by Means of Fluorescent Antibody.* (23849)

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Methods currently employed for classification and identification of yeasts, based upon a combination of morphological and physiological characteristics(1,2) are complex, time-consuming, and require many special media. Conventional serological methods could be applied with advantages of potentially greater specificity and relative simplicity, but difficulties are encountered in preparation of stable and specific antigen systems and a good deal of time and equipment are generally consumed. Several investigators have applied serological technics to classification within the genus *Candida* (3, including a review of the literature; 4), but none of these has reached the status of routine identification procedure.

An entirely new tool for identification of yeastlike organisms became available with development of Coons fluorescent antibody technic(5). Following Goldman's(6) adaptation of the method to differentiation of whole amoeba cells *in vitro,* we became interested in applying it to yeastlike agents of human disease. *Candida albicans,* for example, is commonly identified in the mycology laboratory by formation of characteristic chlamydospores on cornmeal agar. Neither this medium nor any of its numerous substitutes has proved completely reliable for this purpose and, in addition, it is well known that some strains of *C. stellatoidea* form similar spores on these media. Difficulty is often encountered also in precise identification of systemic fungus pathogens in tissue when stained by conventional technics. This applies particularly to the confusion between intergrading forms of *Blastomyces dermatitidis* and *Histoplasma capsulatum.* The present paper deals with the use of specific, fluorescein-labelled anti-*Candida* globulins (conjugates) to identify yeast cells in dried smears.

Materials and methods. Production of fluorescent antibody. Heat-killed saline suspensions (1:200 v/v) of 24 to 48-hour *Candida albicans* cultures (strain Br) on Sabouraud agar were injected intravenously into albino rabbits weighing 5 to 7.5 lb. The immunization schedule was similar to that employed by Benham(7), resulting in serum agglutinin titers of 1:1280 to 1:40,960 against the homologous organism. Globulin fractions of serum from 2 hyperimmunized rabbits, C-1 and C-4, and from a normal rabbit were obtained by ammonium sulfate precipitation. Tube agglutination titers for C-1 and C-4 globulins against *C. albicans* cells were 1:160 and 1:5,120, respectively; protein concentrations (biuret method) 0.43 and 2.72 mg %. The protein content of each was adjusted to 2 mg % for labelling, C-1 being concentrated by evaporation through a dialysis sac in front of fan. Fluorescein isocyanate was synthesized according to the method of Coons and Kaplan(5) and reacted overnight with globulins at 0 to 2°C, C-1 and C-4 globulins being labelled with isocyanate derived from separate lots of amine.† Unreacted fluorescein compounds were removed by dialysis of the conjugate in the cold against phosphate-buffered saline (pH 7). For some staining experiments fluorescent conjugates were absorbed with heterologous yeast cells to increase their specificity. In these cases the sediment from a suspension of fresh yeast cells was resuspended in 1 ml of diluted (1:10) conjugate, the mixture agitated for 2 hours at 37°C on a reciprocating shaker and then refrigerated overnight. Following removal of cells by centrifugation the clear supernatant was employed in staining. *Preparation of slides.* Yeast phases of the diphasic pathogens were cultured upon cystine heart-

* This investigation was supported by research grant from N.I.H., P.H.S.

† Most of the amine was prepared locally by Col. John W. Steedly, The Citadel. The lot from which conjugate C-1 was prepared was kindly contributed by Dr. Morris Goldman, Communicable Disease Center, Atlanta.

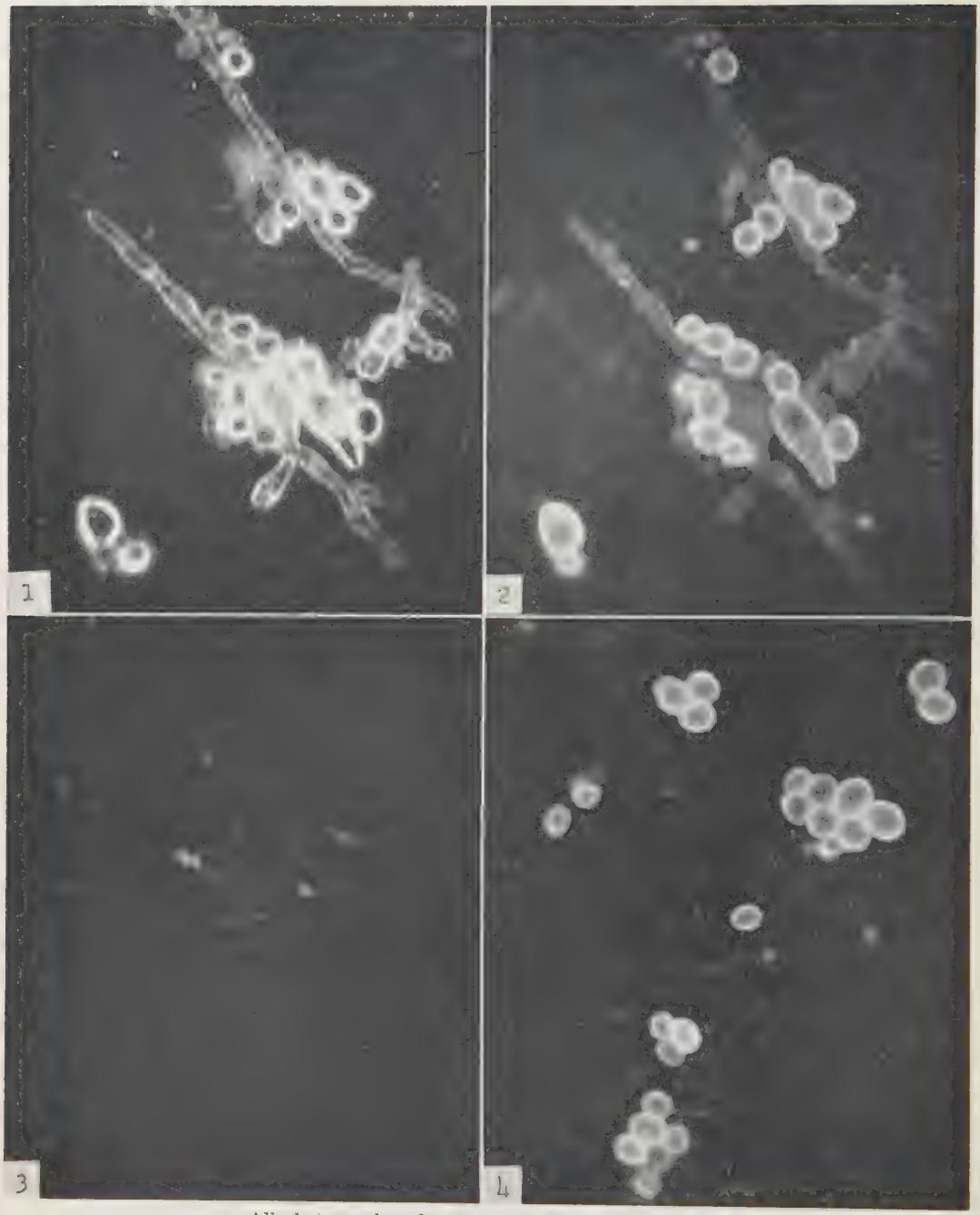
hemoglobin agar slants at 37°C and harvested at 3 to 7 days. All other yeasts were grown on Sabouraud dextrose agar at room temperature for 3 to 4 days. Growth from slants was emulsified directly upon a slide in a drop of distilled water, permitted to air-dry, and gently fixed by heat. Each smear was covered with a drop of labelled globulin and placed in moist chamber at room temperature for 30 minutes. It was then drained, rinsed in 2 changes of buffered saline for 10 minutes, blotted dry, and mounted in a drop of glycerol-saline (9 parts glycerol : 1 part saline, pH 7) under a coverglass. Brightness of fluorescence upon microscopic examination was judged by a system of "plus" symbols similar to that employed by Moody *et al.* (8).

In examination of the slides quantitative readings (estimation of fluorescence) of films prepared from yeast cultures were most consistent when restricted to those areas wherein clusters of cells lay in a single plane rather than those in which scattered cells appeared to be "floating" at different optical levels. *Fluorescence microscopy.* A Reichert Zetopan microscope equipped with cardioid darkfield condenser and Reichert illumination system was used. Illumination consisted of a 30 watt tungsten bulb and a 200 watt, air-cooled, maximum pressure mercury vapor arc lamp, which were instantly interchangeable. A BG 12 3 mm glass filter in front of mercury lamp permitted passage of only near ultraviolet, violet, and some blue light. All this incident light was taken out, after having passed through specimen slide, by a Wratten G gelatin filter fitted into the microscope tube, so that only the secondary, fluorescent rays reached the ocular. Both monocular and binocular heads, fitted with 8X oculars, were employed. Routinely yeast cells were found with a 16mm 10X objective and examined more closely with a 1.8mm 100X apochromat. Photomicrographs were taken on Kodak 35mm Tri-X film, with exposures of 3 seconds for visible light (darkfield) and 30 seconds for fluorescence.

Results. The conjugates were tested against 103 cultures of yeasts or yeastlike organisms, of which 57 were strains of *Candida*. All 28 strains of *Candida albicans* stained 4+ (Fig 1, 2) with a 1:10 dilution of either C-1 or C-4 conjugate, this dilution being employed because it permitted greater specificity of staining without significant sacrifice of sensitivity. No fluorescence was induced by normal globulin conjugate. C-4 1:10 absorbed with cells of *C. parakrusei* (2 successive absorptions with 1:20 v/v suspension of packed cells) stained all but one of the *C. albicans* strains 3+ to 4+, the exception being read as 2-3+. Results of the application of these 3 reagents to the 7 other species of *Candida* are summarized in Table I. It should be pointed out that *C. tropicalis* could not be distinguished from *C. albicans* with this set of conjugates. The remaining species could readily be differentiated from these two with the C-1 stain; likewise with the absorbed C-4, except for 2 strains of *C. stellatoidea*.

Forty-six strains of non-*Candida* yeasts or yeastlike organisms, representing 15 genera and at least 22 species, were exposed to C-1 or C-4 or both. The following species exhibited no fluorescence (0 or ±) (figure in parentheses denotes number of strains tested): *Blastomyces dermatitidis* (12), *B. brasiliensis* (2), *Histoplasma capsulatum* (4), *H. duboisii* (2), *Sporotrichum schenckii* (1), *Cryptococcus neoformans* (2), *C. albidus* (1), *Trichosporon cutaneum* (1), *Trichosporon* sp. (1), *Geotrichum candidum* (3), *Rhodotorula* sp. (4), *Pullularia pullulans* (2), *Hansenula anomala* (1), *H. californica* (1) (ascospores of these two fluoresced, but not the vegetative cells), *Lipomyces starkeyi* (1), *Saccharomyces cerevisiae* (1), *S. veronae* (1) (ascospores fluoresced), *Torulopsis glabrata* (1). One strain of each of the following species exhibited fluorescence of at least 1+ when treated with unabsorbed C-4 (intensity denoted by figure following): *Endomycopsis fibuliger* 1-2+, *Pichia fermentans* 1-3+, *P. polymorpha* 2+, *Hansenula beckii* 2+ (ascospores 4+), *Debaryomyces hansenii* 3-4+. Absorption of the conjugate with *C. parakrusei* eliminated these reactions. Various unidentified cultures of bacteria exposed to the above conjugates exhibited no fluorescence whatsoever.

As a further control on the serological specificity of the observed staining reactions,



All photographs taken at a magnification of 800 ×.

FIG. 1. Mixture of *Candida albicans* and *Histoplasma capsulatum* cells exposed to *C. albicans* (C-4) conjugate for 30 min. Visible light, darkfield.

FIG. 2. Same field as Fig. 1, ultraviolet light. Only the homologous cells fluoresce.

FIG. 3. *C. albicans* exposed simultaneously to C-4 conjugate and unlabelled C-4 serum for one hr. Ultraviolet light. Inhibition of staining.

FIG. 4. *C. albicans* exposed simultaneously to C-4 conjugate and normal serum for one hr. Ultraviolet light. No inhibition of staining.

TABLE I. Fluorescence of *Candida* Species Exposed to Labelled Anti-*C. albicans* Globulins (1:10 Dilution).

Species of *Candida*	No. of strains	Range of fluorescence, each conjugate		
		C-1	C-4	C-4 absorbed
C. tropicalis	8	2+ to 4+	2+ to 4+	2+ to 4+
stellatoidea	7	0 to 1+	2+ to 4+	0 to 3+
parakrusei	3	0 to 2+	2+	0
krusei	4	0	2+ to 4+	0 to 1+
pseudotropicalis	2	0	0 to 2+	0
guilliermondi	4	0 to 2+	± to 4+	0
lipolytica	1		0	

homologous absorption procedures were applied to the C-1 and C-4 conjugates. It was found that a 1:10 dilution of either, when absorbed with an equal volume of a 1:5 v/v suspension of either *C. albicans* or *C. tropicalis,* no longer stained smears of these or any other species of *Candida*. Further, staining of the homologous cells by the unabsorbed conjugates was inhibited by either prior or simultaneous exposure to unlabelled specific serum (Fig. 3, 4).

Some preliminary trials with non-*Candida* conjugates may be reported. Anti-*Histoplasma capsulatum* (H-3) and anti-*Blastomyces dermatitidis* (B-1) globulins obtained from rabbits experimentally infected with the respective organisms were labelled with fluorescein isocyanate as described above. The B-1 conjugates stained yeast cells of both *B. dermatitidis* and *C. albicans* strongly, but exhibited little or no reaction with *Blastomyces brasiliensis* or *H. capsulatum*. H-3 conjugates stained *H. capsulatum* strongly, *B. dermatitidis* to a much lesser extent, and reacted only very weakly with *B. brasiliensis*.

Comments. The fluorescent antibody technic retains the high specificity inherent in serological procedures while offering several advantages over agglutination and precipitation methods for the identification of yeasts. The problem of obtaining a smooth agglutinating antigen is obviated, as are the complex procedures involved in extraction of a soluble and specific precipitin antigen. Large numbers of cells are not required for the reaction, since even a single organism can be identified by stain, and it need not be viable. Additional diagnostic potentials of the fluorescent antibody method for medical mycology are readily apparent. It may be applied, for example, to the serological examination of body fluids by the inhibition procedure described by Goldman(9) for toxoplasmosis and utilized by us herein as a specificity control (Fig. 3, 4). Also, preliminary studies have shown that yeast cells and pseudohyphae of *Candida albicans* may be stained directly in clinical specimens such as tissue sections, smear impressions and material obtained by oral or vaginal swab. This would be of value in differentiating this pathogen from the nonreacting harmless commensals, such as *C. stellatoidea, Cryptococcus,* and *Saccharomyces,* particularly when pseudohyphae are not seen. Our *Candida* conjugates also display no reaction with *Torulopsis glabrata,* whose increasing importance as a possible human pathogen has recently been reported by Wickerham (10). Thus, another potential source of confusion may be eliminated.

Summary. 1) The Coons fluorescent antibody technic is here extended to differentiation and classification of yeasts. The globulin portion of antiserum produced in rabbits against *Candida albicans* was labelled with fluorescein isocyanate and applied as a differential stain to dried smears from various yeastlike cultures. 2) All 28 strains of *C. albicans* tested and all 8 strains of *C. tropicalis* exhibited positive staining reactions with the anti-*C. albicans* conjugates. Twenty-one strains belonging to 6 other species of *Candida,* and 46 strains of yeastlike organisms representing 15 other genera were unstained or only weakly stained, and could readily be distinguished from the preceding 2 species. The staining specificity of high-titered conjugates was greatly increased through absorption with cells of *Candida parakrusei*. 3) Appropriate controls on the serological specificity

of the fluorescent staining, including inhibition and absorption tests, are described. 4) Preliminary results with *Blastomyces dermatitidis* and *Histoplasma capsulatum* conjugates are reported.

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Tyrosine, Iodide, and Human Serum Lipoproteins.* (23850)

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Thyroid active substances such as desiccated thyroid(1,2), thyroxine(3) and l-triiodothyronine(4) have all been shown to have a profound effect on human serum lipoprotein concentrations.

Since tyrosine and inorganic iodine are the only 2 known precursors required for thyroidal synthesis of thyroxine, the effect of administration of tyrosine and of iodide on human serum lipoproteins has been investigated.

Tyrosine study; methods. Serial blood samples were obtained from a group of 16 male hospitalized chronic schizophrenic patients, judged by routine laboratory tests and a physical examination to be free of other disease. None of these patients had received any medication or electroshock therapy for several weeks to months before initiation of the present study. Eleven of these patients, ranging in age from 31-47 years, received a daily dose of 30 g of tyrosine suspended in glass of water daily for 18 weeks; the remaining 5 patients, ranging in age from 29-48 years, served as a control group receiving no medication. Five blood samples were obtained in all patients during a 6 month interval preceding tyrosine administration, to serve as a base line, 7 samples were drawn during the 18-week period of tyrosine intake and 3 samples in a 6-week period following the last dose of tyrosine. Serum lipoprotein(5) and total serum cholesterol analyses(6) were done on all blood samples and body weight, blood pressure and pulse rate were recorded for each patient at the time of venipuncture.

Results of complete low density serum lipoprotein analyses and of cholesterol determinations in 240 blood samples and of body weight, blood pressure and pulse rate data are summarized in a greatly condensed manner in Table I. Analysis of all the data shows that a daily dose of 30 g of tyrosine, estimated to be approximately equivalent to 8-10 fold the normal dietary intake(7), has no effect on

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TABLE I. Effect of Tyrosine* on Serum Lipoprotein and Total Serum Cholesterol Concentrations and on Body Weight, Blood Pressure and Pulse Rate.

	Serum lipoprotein concentrations: mg/100 ml of serum								Total serum cholesterol, mg/100 ml	
	S_f 0-12		S_f 12-20		S_f 20-100		S_f 100-400			
	P†	C†	P	C	P	C	P	C	P	C
Pre‡	306	372	28	38	71	89	34	51	206	238
On§	289	367	27	34	74	103	31	77	192	230
Off‖	282	374	25	39	67	103	34	55	186	232

	Body wt		Systolic B.P.		Diastolic B.P.		Pulse rate	
	P	C	P	C	P	C	P	C
Pre	146	150	133	133	81	85	96	83
On	146	152	139	144	86	86	98	90
Off	149	155	135	137	83	83	94	85

* 30 g/day.
† P = patients, C = controls.
‡ All "pre" values represent mean of 5 samples
§ " "on" " " " " 7 "
‖ " "off" " " " " 3 "
} obtained in each of 5 control and 11 treated patients.

serum lipoproteins, body weight, blood pressure and pulse rate. No side effects such as nausea, vomiting, diarrhea or other untoward reactions occurred nor was there any consistent effect on psychiatric status.

Iodide study; methods. The effect of oral intake of potassium iodide on serum lipoprotein concentrations was studied in 5 ambulatory office patients followed for several years because of associated illness. Two patients had a diagnosis of xanthoma tuberosum, one xanthoma tendinosum, one CVA with hypertension and one myocardial infarction. Each patient received a daily dose of 30 mg of potassium iodide for a period varying from 10 to 36 weeks during which 5 to 15 blood samples were obtained for serum lipoprotein and cholesterol analyses. Mean value for serum lipoprotein and cholesterol concentrations before and during potassium iodide administration are collected in Table II.

Results. The data show that potassium iodide does not produce any consistent changes in serum lipoprotein distribution or in cholesterol concentration in the patients studied. The only apparently significant changes observed occurred in the S_f 100-400 concentrations and in total serum cholesterol levels in one patient with a diagnosis of xanthoma tuberosum. Previous experience, however, has shown that the S_f 100-400 concentrations in

TABLE II. Effect of Potassium Iodide on Serum Lipoprotein and Total Serum Cholesterol Concentrations.

	Time interval (wk)	Serum lipoprotein conc., mg/100 ml serum				Cholesterol, mg/100 ml	Diagnosis	Age, sex
		S_f 0-12	S_f 12-20	S_f 20-100	S_f 100-400			
Off (2)*		96	70	525	801	604	Xanthoma	47 ♂
On (9)	16	110	89	582	488	417	tuberosum	
Off (2)		141	107	361	34	268	Xanthoma	41 ♀
On (8)	14	154	121	355	51	270	tuberosum	
Off (4)		439	30	58	20	247	CVA, hyper-	64 ♂
On (13)	36	450	40	68	24	259	tension	
Off (7)		356	38	71	22	223	Myocardial in-	55 ♂
On (15)	35	343	42	67	16	223	farction (old)	
Off (7)		1135	118	44	0	618	Xanthoma	52 ♀
On (5)	10	1071	106	36	0	567	tendinosum	

* All lipoprotein and cholesterol data represent mean values derived from number of blood samples indicated in parentheses.

patients with xanthoma tuberosum is frequently extremely labile so that no special significance can be attributed to these changes seen in only one of 2 patients and based on limited data.

Conclusions and summary. 1) Tyrosine and potassium iodide do not have any effect on serum lipoprotein and total serum cholesterol concentrations. 2) These findings indicate that in clinically euthyroid patients neither substance appears to be a limiting factor in thyroidal synthesis of thyroid hormones from tyrosine and iodide.

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Histoplasmin-Latex Agglutination Test. II. Results with Human Sera.* (23851)

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Previous studies from this laboratory(1,2) have shown that polystyrene latex particles sensitized with histoplasmin can be used in a simple tube agglutination technic to detect antibody against *Histoplasma capsulatum* of infected or immunized animals. The latex particles were employed in a manner similar to that utilized in the collodion agglutination test for histoplasmosis(3-5). The purpose of the present report is to compare agglutination test results obtained with latex and collodion particles in the presence of human sera.

Materials and methods. Procedures used in preparation of materials and in performance of histoplasmin-latex agglutination tests were described in detail previously(2). In brief, a suspension of latex particles† is mixed with the previously-determined optimal dilution of histoplasmin, and kept at room temperature 1-2 hours. This antigen is then added to serial 2-fold dilutions of serum. After incubation for 2 hours at room temperature, test tubes are centrifuged at 2000 rpm for 5 minutes, and read after gentle flicking or tapping of the tubes. Reactions are graded from 1+ to 4+, depending on size of agglutinated particles and opacity of the supernatant, as in any agglutination reaction. In previous work with animal sera(2), agglutinations were read with the aid of a concave microscope mirror. This practice has been discontinued, however, and in the present study all tests were read by gross examination of the tube held in front of an appropriately shaded light (Fig. 1). The technic of the histoplasmin-collodion agglutination test has been described previously(3).

Results with random human sera. To obtain informaton as to specificity of the histoplasmin-latex test, and the frequency with which so-called "normal" human serum samples would yield detectable reactions, the test was performed on 204 random specimens originally submitted to the Ohio Department of Health Laboratory for routine syphilis serol-

* These studies were supported in part by Research Grant from NIH, USPHS.

† Dow Chemical Co., Midland, Mich.

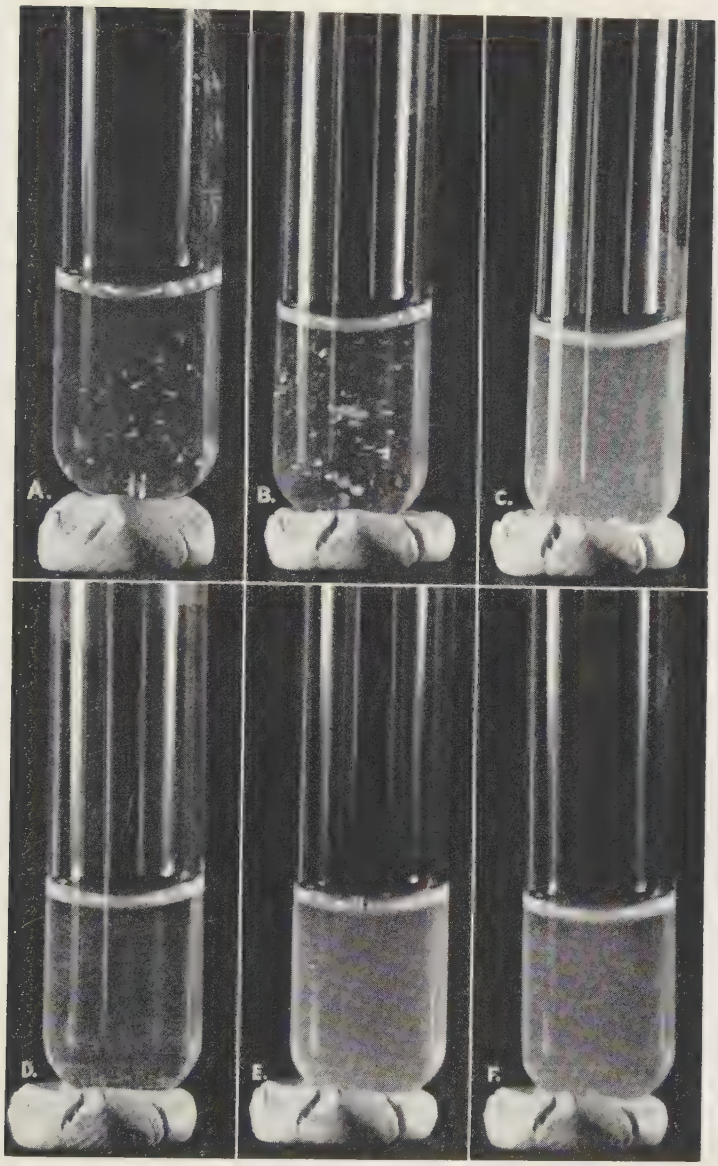

FIG. 1. A histoplasmin-latex agglutination test. A. 4+ latex agglutination. B. 4+ collodion agglutination. C. 2+ latex agglutination. D. 2+ collodion agglutination. E. Negative latex agglutination. F. Negative collodion agglutination.

ogy. Only one of the 204 sera exhibited 4+ agglutination; this sample was positive also by the collodion test, suggesting that this patient had histoplasmosis. Latex agglutination reactions of 3+ intensity were observed in 4 samples (2%) at only a 1:5 serum dilution, while 55 (26.9%) showed 1+ or 2+ reactions at serum dilutions of 1:5 and 1:10. These results are presented in Table I.

TABLE I. Histoplasmin-Latex Agglutination Reactions with 204 Human Sera Originally Submitted for Syphilis Serology.

Intensity of reaction	Serum dilution 1:5	1:10	1:20	Total	%
4+	—	1	—	1	0.5
3+	4	—	—	4	2.0
1+ – 2+	40	15	—	55	26.9
			Total	60	29.4

TABLE II. Histoplasmin-Latex and Histoplasmin-Collodion Agglutination Reactions with 208 Human Sera Originally Submitted for Histoplasma Serology.

Intensity of reaction	Latex test: Serum dilution 1:5	1:10	1:20	Total	%	Collodion test: Serum dilution 1:5	1:10	1:20	Total	%
4+	—	1	—	1	.5	—	—	—	—	.0
3+	1	1	—	2	1.0	11	6	—	17	8.2
1+–2+	56	18	1	75	36.0	36	29	—	65	31.2
			Total	78	37.5				82	39.4

TABLE III. Histoplasmin-Latex and Histoplasmin-Collodion Agglutination Reactions with 42 Positive Human Sera.

Type of test	Intensity of reaction	Serum dilution 1:5	1:10	1:20	1:40	1:80	1:160	1:320	Total
Latex	4+	5	8	11	9	4	1	—	38
	3+	1	3	—	—	—	—	—	4
	1+–2+	—	—	—	—	—	—	—	0
								Total	42
Collodion	4+	5	12	7	9	8	1	—	42
	3+	—	—	—	—	—	—	—	0
	1+–2+	—	—	—	—	—	—	—	0
								Total	42

In addition, 208 serum specimens from University Hospital patients with various diseases other than histoplasmosis, were studied by the latex agglutination test. These sera had been considered negative because of absence of histoplasma infection, and their failure to give 4+ reactions in the collodion agglutination test. One of these 208 samples (0.5%) showed 4+ reactivity in the latex test. Reactions of 3+ intensity were seen in only 1 sample (0.5%) at a serum dilution of 1:5 and in only 1 sample (0.5%) at a serum dilution of 1:10, while 75 (36%) showed 1+ or 2+ reactions in serum dilutions between 1:5 and 1:20. In the collodion particle test, 17 (8.2%) of these same 208 samples yielded 3+ reactions at serum dilutions of 1:5 and 1:10, while 65 (31.2%) gave 1+ or 2+ reactions at dilutions of 1:5 and 1:10. Comparative results of latex and collodion agglutination tests on the 208 sera are summarized in Table II. It should be mentioned that although the number of reactive sera was approximately the same as measured by each of the 2 tests, individual serum samples did not react the same in the 2 tests. In many cases, specimens yielded 1+ or even 2+ reactions at serum dilutions of 1:5 and 1:10 in the latex test and were completely negative by collodion agglutination. The reverse was true in approximately the same number of cases.

Results with positive human sera. Comparative studies of histoplasmin-latex and histoplasmin-collodion agglutination tests were carried out with 42 sera from patients with histoplasmosis. Twenty-five were from patients in University Hospital; the remainder were kindly supplied by Dr. John Procknow,‡ Miss Charlotte Campbell,§ and Mr. Arthur H. Bauer.‖ Table III summarizes results obtained with the 42 samples in both tests. All of the 42 sera gave 4+ reactions in the collodion test at serum dilutions of 1:5 or higher, while 38 of 42 yielded 4+ reactions in the latex test; 3 of the remaining 4 sera gave 3+ reactions at 1:5 and 1:10 and the 4th at 1:5 only. These latter 4 sera (Nos. 31, 33, 36, 38) are included in Table IV which depicts the results obtained with 12 less strongly positive sera, and compares relative sensitivity of the 2 tests at a lower level of positivity. These data suggest that the collodion agglutination is slightly more sensitive than the latex procedure.

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§ Walter Reed Army Medical Center.

‖ Ohio State Department of Health Laboratories.

TABLE IV. Histoplasmin-Latex and Histoplasmin-Collodion Agglutination Reactions with Moderately Positive Human Sera.

Specimen No.	Latex Serum dilution 1:5	1:10	1:20	1:40	1:80	1:160	C*	Collodion Serum dilution 1:5	1:10	1:20	1:40	1:80	1:160	C*
31	3	2	2	1	—	—	—	4	4	2	1	—	—	—
32	4	3	2	1	—	—	—	4	4	3	2	1	—	—
33	3	3	2	1	—	—	—	4	4	3	3	2	1	—
34	4	3	2	1	—	—	—	4	3	2	1	—	—	—
35	4	3	3	2	1	—	—	4	4	3	2	1	—	—
36	3	3	2	1	—	—	—	4	3	2	1	—	—	—
37	4	4	2	1	—	—	—	4	4	3	2	1	—	—
38	3	3	2	1	—	—	—	4	4	3	2	1	—	—
39	4	4	4	2	1	—	—	4	3	2	1	—	—	—
40	4	4	4	3	1	—	—	4	3	3	2	1	—	—
41	4	3	2	1	±	—	—	4	3	2	1	—	—	—
42	4	3	2	2	1	—	—	4	4	3	2	1	—	—

* Control—Serum 1:5 and particles without histoplasmin.

Summary. This laboratory has now had 10 years experience with the collodion agglutination test for histoplasmosis(3-9). We use it routinely in screening for active histoplasmosis. The test itself is simple to perform and read. However, smaller laboratories or institutions with lesser demand for serologic studies for histoplasmosis find the preparation of collodion particles too time-consuming for their purposes. Commercially-prepared latex particles show similar results as collodion particles when both are sensitized with histoplasmin. It is suggested from these studies that, as in the collodion agglutination test, a 4+ reaction at 1:5 dilution or higher is compatible with active histoplasmosis. In 4 instances 3+ latex agglutinations were observed in sera showing 4+ collodion tests. Thus, at the present time, 3+ agglutinations with latex are placed in the "suspicious" category. As with collodion agglutinations, 1-2+ reactions are considered negative.

Studies are in progress to evaluate further the use of histoplasmin-latex agglutinations in serologic diagnosis of histoplasmosis.

The authors are indebted to Joann Sparks for technical assistance.

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Effect of Certain Amino Acids and Peptides on Hyaluronidase Production by *Staphylococcus aureus*.* (23852)

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Rogers(1) devised a semisynthetic medium for production of hyaluronidase by a number of bacteria, including *Staphylococcus aureus*. This medium consisted of acid hydrolyzed

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casein, cystine, tryptophan, thiamin, nicotinic acid, and the necessary salts to keep the medium well-buffered, since hyaluronidase synthesis ceases when pH of culture falls below 6.0. Although *S. aureus* has been cultivated in chemically defined media(2,3), synthesis of hyaluronidase in a completely synthetic medium has not been described. During investigation on synergistic infections produced by concerted action of anaerobic streptococci and *S. aureus* injected into the rabbit skin, it was observed that hyaluronidase containing cell-free filtrates of an *S. aureus* brain heart infusion culture were capable of enhancing anaerobic streptococcal infections(4). To identify and characterize further the factors in this staphylococcal filtrate responsible for this enhancement phenomenon, a synthetic medium was devised for growth and hyaluronidase production by this strain of *S. aureus*. Certain amino acids appear essential for enzyme synthesis but not for growth and certain dipeptides stimulate both growth and hyaluronidase production.

Materials and methods. The strain of *S. aureus,* designated as SAB2, was originally isolated from a human blood culture. This hemolytic, coagulase positive organism was maintained on sheep-blood agar by weekly transfer. Brain heart infusion broth (Difco) and heart infusion broth (Difco) served as reference nonsynthetic media. Good growth of this organism was obtained in a synthetic medium of pH 7.5 containing per 100 ml of medium: L-arginine hydrochloride, 40 mg; L-cystine, 40 mg; DL-valine, 40 mg; L-proline, 20 mg; DL-leucine, 20 mg; DL-phenylalanine, 40 mg; glycine, 20 mg; L-histidine hydrochloride, 40 mg; L-aspartic acid, 20 mg; L-lysine hydrochloride, 40 mg; L-glutamic acid, 10 mg; DL-tryptophan, 40 mg; L-tyrosine, 20 mg; nicotinic acid, 2 μg; thiamin hydrochloride, 2 μg; glucose, 1 g; $MgSO_4 \cdot 7H_2O$, 1 mg; $Mn\ SO_4\ H_2O$, 1 mg; $FeSO_4 \cdot 7H_2O$, 1 mg; K_2HPO_4, 500 mg; KH_2PO_4, 500 mg; and NaCl, 500 mg. This medium was sterilized by filtration through a Selas 02 candle. Solutions of glycyl-L-tryptophan and glycyl-L-tyrosine were sterilized separately by filtration and added to medium to give a concentration of 70 mg/100 ml of medium. In experiments that follow "complete synthetic medium" refers to the chemically defined medium outlined above minus the 2 dipeptides. The adequacy of media for growth and hyaluronidase production was determined by inoculation of 50 ml Erlenmeyer flasks containing 25 ml of media with 0.05 ml of a suspension containing 3 x 10^9 twice-washed cells/ml as determined by a McFarland nephelometer. All cultures were incubated on a rotary shaker at 37°C. The inocula were collected from a 24-hour culture in heart infusion broth. Bacterial growth was estimated on a Klett-Summerson photoelectric colorimeter using red filter No. 66. An uninoculated tube of medium was used to zero the instrument. Hyaluronidase concentrations of culture supernates were determined by the turbidimetric method(5) using potassium hyaluronate isolated from human umbilical cord(4) as substrate.

TABLE I. Effect on Growth and Hyaluronidase Production of Omitting Amino Acids from a Synthetic Medium for *Staphylococcus aureus.*

Amino acids omitted	Growth*	Hyaluronidase (TRU/ml)
None	100	20
Lysine, arginine, cystine	37	0
Glycine, valine, leucine	4	0
Phenylalanine, tyrosine, tryptophan	89	0
Aspartic acid, glutamic acid, histidine, proline	7	0
Phenylalanine	112	16
Tyrosine	108	0
Tryptophan	82	0
Control: Heart infusion broth	233	40

* Expressed as % of turbidity of 24-hr culture in complete synthetic medium. 100% approximates colorimeter reading of 240.

Results. *Staphylococcus aureus,* strain SAB2, synthesized 40-50 turbidity reducing units (TRU) of hyaluronidase/ml of culture when grown 24 hours in either brain heart infusion or heart infusion medium. Although only one-half this amount of enzyme was produced in the complete synthetic medium (16-20 TRU), there was a similar reduction in bacterial growth (Table I).

By omission of amino acids in groups, as recommended by Lederberg(6), it was found (Table I) that certain groups appeared essential for optimal growth with the exception of

TABLE II. Effect of Peptides and Varying Concentrations of Amino Acids on Growth and Hyaluronidase Production by *Staphylococcus aureus*.

Medium	Growth*	Hyaluronidase (TRU/ml)
Complete synthetic, 1× full complement of amino acids	100	16
Complete synthetic + glycyl-L-tyrosine and glycyl-L-tryptophan	198	32
Complete synthetic, 2× tyrosine, tryptophan and glycine	126	16
Complete synthetic, 2× full complement of amino acids	148	20

* Expressed as % of turbidity of 24-hr culture in complete synthetic medium.

phenylalanine, tyrosine, and tryptophan. The simultaneous elimination of these amino acids, however, abolished hyaluronidase production. In the absence of each of these 3 amino acids singly, growth was comparable in all cases to that in the complete synthetic medium, but enzyme production could not be detected in the absence of tyrosine or tryptophan. Omission of phenylalanine alone, however, had no effect on hyaluronidase production by this organism. Although a moderate amount of growth took place in the medium lacking lysine, arginine, and cystine, hyaluronidase could not be detected in this culture. It seems quite possible that small quantities of hyaluronidase were present (<0.5TRU) but that the method of assay is not sensitive enough to determine these lower concentrations with certainty.

Omission of single amino acids demonstrated that glycine was indispensable for growth of this organism and that valine and leucine, although not completely essential for growth, were highly stimulatory. With the knowledge that glycine was essential for growth, additions to the synthetic medium of dipeptides containing glycine and the 2 amino acids essential for hyaluronidase production were tested for their possible stimulatory effect on growth and hyaluronidase production. The effect of the addition of these peptides to the synthetic medium on growth and hyaluronidase production was compared with the response observed in the complete synthetic medium, the complete synthetic medium containing twice the usual concentrations of the amino acids glycine, tyrosine, and tryptophan, and the complete synthetic medium containing twice the usual concentrations of all amino acids (Table II). Although growth was stimulated in most cases by increase in free amino acids, the greatest enhancement of growth and hyaluronidase production took place in the synthetic medium supplemented with the dipeptides. A further experiment demonstrated that addition of either peptide alone resulted in the same stimulation of growth and enzyme production.

By sampling a continuous culture at various intervals of time, it was shown (Table III) that hyaluronidase synthesis and growth have a considerable lag period in the synthetic medium as compared with brain heart infusion. In the synthetic medium, bacterial turbidity was first detected at about 3 hours and it appears that enzyme production did not begin until the 7th hour and then increased as growth developed. Both growth and hyaluronidase production were detected in supernates from the nonsynthetic medium after a shorter lag period (3 hours) and both increased considerably in the next 24 hours. It seems reasonable to assume that hyaluronidase production reaches a limiting peak between 12 and 24 hours since samples taken after 24 hours failed to detect an increase in hyaluronidase in either medium.

Discussion. Lower production of hyaluroni-

TABLE III. Hyaluronidase Production at Various Time Intervals during a Continuous Culture Experiment.

	Complete synthetic + peptides*		Brain heart infusion*	
Time, hr	Growth†	Hyaluronidase, TRU/ml	Growth†	Hyaluronidase, TRU/ml
1	0	0	0	0
2	8	0	18	0
3	12	0	128	4
4	12	0	204	32
5	—	—	264	32
6	28	0	300	48
7	32	0.5	345	—
11	135	8	415	48
12	200	20	428	48
24	345	38	545	48

* 250 ml Erlenmeyer flask containing 100 ml media incubated on rotary shaker at 37°C.
† Colorimeter reading.

dase by *S. aureus* in a chemically defined medium than in nonsynthetic media demonstrated the necessity for additional nutritional factors for optimal production of this enzyme. Rogers(1) has reported similar results. The same situation has been reported for production of other enzymes such as lecithinase by *Clostridium perfringens*(7). Tyrosine, phenylalanine, and tryptophan were dispensable for optimal growth of *S. aureus*, strain SAB2, in the synthetic medium used. This result agrees with previous work demonstrating that these 3 amino acids are dispensable for growth of a number of strains of staphylococci(3). However, this organism required tryptophan and tyrosine for synthesis of hyaluronidase. Glycine was completely essential for growth. Furthermore, an increase in growth and hyaluronidase production was noted when the synthetic medium was supplemented with either glycyl-L-tryptophan or glycyl-L-tyrosine. This may indicate that the peptides enhance growth and enzyme production by supplying the essential amino acids in a more utilizable form(8).

Summary. *Staphylococcus aureus*, strain SAB2, synthesized hyaluronidase in a chemically defined medium only in the presence of tyrosine and tryptophan, even though these amino acids were not required for optimal growth. Glycine was indispensable for growth of this organism. Supplementation of the synthetic medium with glycyl-L-tyrosine or glycyl-L-tryptophan enhanced both growth and hyaluronidase production.

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Liver Ketogenesis: Role of Glucose-Cyclo-Acetoacetate on Ketogenesis From Fatty Acids and Pyruvate. (23853)

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After having shown(1-7) that acetoacetate plays an important role in development of experimental diabetes, we noticed that compounds formed by condensing acetoacetate with glucose are not only non-diabetogenic but act as an important agent in prevention of various kinds of experimental diabetes induced by alloxan, dehydroascorbic acid and acetoacetate(8-12). Because unlike other diabetogenic substances, acetoacetate is a normal metabolite, it is reasonable to assume that gradual accumulation of acetoacetate is responsible for progressive development of clinical diabetes. Such accumulation may be caused either by excessive hepatic ketogenesis (formation of acetoacetate) or by decreased extra hepatic utilization. But it has been demonstrated by Chaikoff *et al.*(13) that tissues of diabetic animals can oxidize ketone bodies as readily as those of normal animals. Raper and Smith(14) are also of the opinion that ketosis must be due, mainly, to this overproduction of ketone bodies by liver rather than to its underutilization by extra-hepatic tissues. Jowett and Quastel(15) found that liver slices form 10 to 40 times more ketone bodies than other organs and Edson(16) has shown that when butyric and crotonic acids

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(Na salt) are added to liver slices, production of acetoacetate is greatly increased. Fatty acids, when added to liver slices are known to be converted to acetyl COA through B-oxidation(17). Pyruvate is also known to be converted to acetyl COA before it is oxidized in the Krebs' Cycle(18). Acetyl COA, derived either from fatty acids or from pyruvate, undergoes condensation to form acetoacetyl COA, which in turn gives rise to acetoacetate(19). Administration of insulin to diabetic animals has been reported by Stadie *et al.*(20) to check rapid production of ketones by the liver. Because glucose-cyclo-acetoacetate prevents onset of acetoacetate-induced and other experimental diabetes, it has been thought worthwhile to see if this compound or any of its derivatives may be helpful in arresting rapid formation of ketone bodies by liver.

We also studied utilization of pyruvate in presence of glucose-cyclo-acetoacetate and its hydrolyzed product.

Methods. Male albino rats of 150 g on adequate normal diet containing 4% McCollum Davis's Salt Mixture, were used. Rats were fasted 18 hours before sacrifice by decapitation. Liver was quickly transferred to ice cold saline. Slices were cut freehand using razor blade. Tissue preparation and incubation methods were described by Umbreit *et al.*(21) in conventional Warburg Vessels (15 cc) and pure oxygen as gas phase was used. Vessels containing Ringer Phosphate buffer (pH 7.2) total volume of fluid in each vessel 3.2 ml were equilibrated 10 minutes at 37.5°C and readings for oxygen uptake were taken during 2 hour incubation period. Then acetoacetate was estimated by aniline citrace method as described by Edson(16). Pyruvate was estimated by method of Friedman *et al.* (22). Preparation of crystalline condensation product was done according to the method of West(23), modified by Nath *et al.*(24). It was dissolved in 2 cc/g 2N NaOH by heating in boiling water-bath for 2 minutes and thus converted to its sodium salt, and was brought to pH 7.2 with HCl, before use. Condensation product was hydrolyzed in 2N HCl for 10 minutes by keeping in the water-bath. The ether soluble fraction was removed and aqueous solution neutralized with NaOH and adjusted to pH 7.2 prior to use. In control vessels, the solution obtained after neutralizing same amount of NaOH was neutralized with HCl to pH 7.2. This solution will compensate excess of sodium chloride present along with Na salt of condensation product or its hydrolysate.

Results. Table I indicates oxygen uptake and production of acetoacetate by liver slices from fatty acids and B-hydroxybutyrate. When glucose-cyclo-acetoacetate or its hydrolysate was added, the acetoacetate produced was much less than that in the normal. Oxygen consumption was, however, slightly depressed. Fatty acids are, therefore, oxidised by liver in presence of this substance in such a way that excess production of acetoacetate is checked. It is well established that acetoacetate and B-hydroxybutyrate are interconvertible in liver and they are not oxidized to any appreciable extent in that organ. When acetoacetate was added to liver slices, its recovery in presence of glucose-cyclo-acetoacetate was, however, not found to be much affected. So also the conversion of B-hydroxybutyrate to acetoacetate is not appreciably affected by these substances. This indicates that glucose-cyclo-acetoacetate or its hydrolysate has no effect on interconversion of acetoacetate to B-hydroxybutyrate. Therefore, acetoacetate formed in presence of liver may be taken as the index of ketogenesis by liver.

It is evident from data in Table II that utilization of pyruvate, as estimated by its disappearance from the incubation media, was much greater in presence of glucose-cyclo-acetoacetate (hydrolyzed). Oxygen uptake was not found to increase considerably and acetoacetate formed, both endogenous and exogeneous, was checked. It is reasonable to assume, therefore, that extra-pyruvate utilized by liver (Table II) and diaphragm (Table III) in presence of these compounds might be used for lipogenesis or glycogenesis, or may be reduced to lactic acid by tissues. This substance was not found to have any effect on the endogenous formation of pyruvate by liver and diaphragm.

Table IV shows that the depressed utiliza-

TABLE I. Acetoacetate Produced and Oxygen Consumed by Liver Slices in Presence of Fatty Acids. (Medium Ringer phosphate buffer, pH 7.2 Temp., 37.5°C.)

No. of rats	Substrate	Substrate conc.	Glucose-cyclo-acetoacetate .016 M	Glucose-cyclo-acetoacetate (hydrolysed) .016 M	Oxygen uptake per 100 mg wet wt of tissue in 2 hr	Acetoacetate produced* per 100 mg wet wt of tissue in 2 hr
5	Crotonate	Nil			236 ± 23	22 ± 8
		.017 M			404 ± 27	128 ± 20
		"	Added		348 ± 35	80 ± 15
		"		Added	330 ± 20	65 ± 11
9	Butyrate	Nil			230 ± 23	22 ± 8
		.017 M			320 ± 23	88 ± 11
		"	Added		300 ± 30	22 ± 9
		"		Added	290 ± 25	20 ± 8
3	Acetate	Nil			269 ± 23	15 ± 5
		.02 M			304 ± 20	60 ± 16
		"	Added		300 ± 30	32 ± 9
		"		Added	281 ± 20	29 ± 9
3	B-hydroxybutyrate	Nil			260 ± 20	11 ± 2
		.01 M			280 ± 24	148 ± 20
		"	Added		280 ± 25	134 ± 8
		"		Added	285 ± 24	132 ± 7
	Acetoacetate	Nil			260 ± 20	11 ± 2
		.0033 M			232 ± 20	76 ± 11
		"	Added		178 ± 21	86 ± 13
		"		Added	175 ± 18	81 ± 14

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.

TABLE II. Acetoacetate Produced and Pyruvate Utilized by Normal Rat Liver Slices.

No. of rats	Glucose-cyclo-acetoacetate (hydrolysed) .016 M	Pyruvate .008 M	Glucose .006 M	μl of oxygen uptake	Acetoacetate* produced	Pyruvate disappeared in γ
				per 100 mg wet wt of tissue in 2 hr		
9				194 ± 14	40 ± 16	325 ± 5
9	Added			232 ± 23	18 ± 1	320 ± 7
12		Added		332 ± 19	100 ± 26	2140 ± 90
12	Added	"		355 ± 25	45 ± 3	2490 ± 80
6		"	Added	201 ± 15	61 ± 5	2000 ±100

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.

TABLE III. Acetoacetate Produced and Pyruvate Utilized by Normal Rat Diaphragm.

No. of rats	Glucose-cyclo-acetoacetate (hydrolysed) .016 M	Pyruvate .008 M	Glucose .006 M	μl of oxygen uptake	Acetoacetate* produced	Pyruvate disappeared in γ
				per 100 mg wet wt of tissue in 2 hr		
9				200 ± 19	Negligible	200 ± 10
9	Added			207 ± 17	"	200 ± 10
12		Added		253 ± 23	16 ± 2	891 ± 30
12	Added	"		319 ± 30	14 ± 2	982 ± 45
6		"	Added	216 ± 4	14 ± 2	530 ± 25

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.

tion of pyruvate by liver slices of alloxan diabetic rats is restored to normal value in presence of glucose-cyclo-acetoacetate (hydrolysate). Villee and Hastings(25) observed less utilization or pyruvate by the diabetic rats' diaphragm, and this was restored to normal level by addition of insulin *in vitro* (5 units/cc). Glucose has not been shown to have any effect on utilization of pyruvate by diaphragm and liver. (Tables, II, III).

Summary. (1) Glucose-cyclo-acetoacetate and its hydrolysate prevented ketogenesis by

TABLE IV. Acetoacetate Produced and Pyruvate Utilized by Liver Slices from Diabetic Rats.†

No. of rats	Glucose-cyclo-acetoacetate (hydrolysed) .016 M	Pyruvate .008 M	μl of oxygen uptake	Acetoacetate* produced	Pyruvate disappeared in γ
			——per 100 mg wet wt of tissue in 2 hr——		
3			128 ± 10	24 ± 3	260 ± 10
3	Added		162 ± 10	15 ± 4	260 ± 11
3		Added	174 ± 11	60 ± 7	1970 ± 30
3	Added	"	139 ± 7	20 ± 10	2155 ± 50

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.
† Alloxan diabetic rats (2 wk)—urine sugar 3%. Air was used as gas phase in vessels.

liver. (2) These substances cause more utilization of added pyruvate, both by liver slices and diaphragm. (3) They have no effect on interconversion of acetoacetate and B-hydroxybutyrate.

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Harmonic Analysis of the Ballistocardiogram. (23854)

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The two mass analysis upon which modern ballistocardiography is based can only be verified for low frequencies(1,2). Above approximately 15 CPS, platform, trunk and limbs no longer move in unison, and the influence of the coupling between heart and skeleton is markedly increased(3,4). On the other hand, frequencies up to 35 CPS may be required to distinguish sequential physiologic events(5, 6). It is therefore important to determine the ballistocardiographic frequency spectrum undistorted by body resonance. In this report

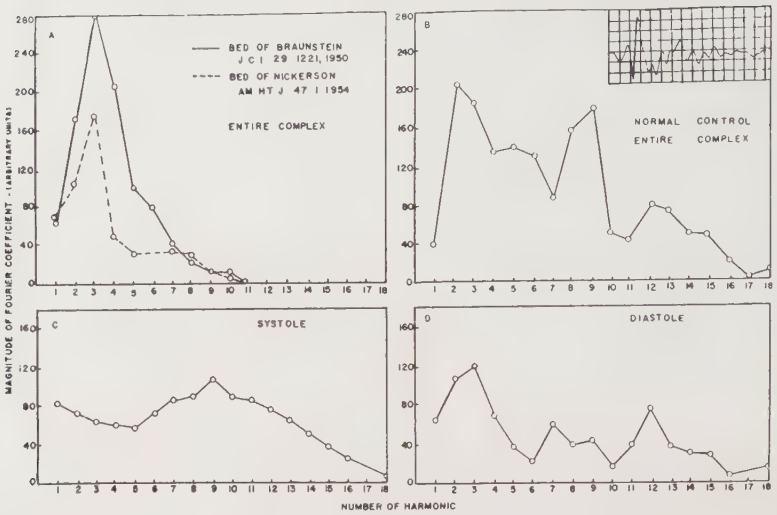

FIG. 1. Fourier analysis of ballistocardiograms recorded with high and low frequency beds (A), and an ultra-low-frequency bed (B, C, and D).

Fourier analyses of high, low, and ultra-low frequency ballistocardiograms are compared, and contrasted with Fourier analyses of aortic flow pulses(7,8). Since the ultra-low-frequency system employed has a theoretically flat frequency response to 35 CPS, the data indicate the relative magnitude of reaction forces between 1 and 20 CPS(2).

Methods. The ballistocardiograph consists of a rigid 16 lb platform suspended with appropriate damping to a natural frequency of 0.5 CPS. Platform acceleration is measured directly, and the band 0.5-25 CPS selected with a Krohn-Hite filter model 330A; filter cut-off slope is proportional to $1/f^2$(2,5). A periodic time function can be represented as the sum of sinusoidal waves of various amplitudes, phase relations and frequencies, in the case of the ballistocardiogram the heart rate and its integer multiples. Such a breakdown is called a Fourier analysis. Twelve by 20 inch photographic enlargements of representative beats recorded from each of 5 normal subjects were examined with a planimetric harmonic analyser, and Fourier coefficients for harmonics 1-25 were determined. Systolic and diastolic deflections are originated by separate physiologic events and involve different anatomic structures(5,6). Frequency spectra were therefore obtained for systole and diastole individually as well as for the entire cycle. To this end systole, diastole and the complete cycle were separately traversed with the analyser, retaining the full cycle length as the fundamental. Diastole was assumed to begin with the ballistic deflection coinciding with the end of the electrocardiographic T wave. This approximation has been validated by subsequent experiments in which the ballistocardiogram and electrocardiogram were recorded simultaneously with central flow and pressure pulses(5,6). The spectrum of the entire complex is not the sum of its systolic and diastolic components as measured because of phase conditions.

Results. Fourier analyses of high and low frequency displacement ballistocardiograms obtained from references 7 and 8 are replotted as Fig. 1A. Both exhibit resonance at 3 CPS, the natural frequency of the unrestrained body moving on its panniculus. Even twice differentiating these curves does not bring out significant components above 10 CPS. Both Nickerson and Braunstein appreciated the fact that their records were distorted by the vibration properties of the body, and Nickerson correctly concluded that forces to at least 11 CPS were important to the acceleration record(7,8).

Fourier analyses of an ultra-low-frequency ballistocardiogram are presented in Fig. 1B, C, and D, together with the oscillographic record from which the data were derived. Note that the frequency spectrum of systolic

forces, Fig. 1C, exhibits no 3-cycle peak. Since resonance is independent of the origin of the driving forces, such a peak should appear in the systolic spectrum if the 3-cycle diastolic peak were due to resonance. Significant frequency components exist to at least 20 CPS in recordings from all 5 subjects, a finding which would be improbable if resonance were not eliminated. We therefore conclude that the ultra-low-frequency ballistocardiogram is indeed free of the vibration properties of the subcutaneous tissues, and that the band 0.5-20 CPS is the minimum, though not necessarily optimum, frequency range over which the acceleration ballistocardiogram must be recorded without phase or amplitude distortion.

Systolic and diastolic spectra are completely different and vary independently during hemodynamic change, indicating that diastolic vibrations represent new forces rather than a dying out process. For example, nitroglycerine, 0.32 mg sublingually, increases heart rate and ballistic amplitude and decreases arterial pressure and the magnitude of high frequency reaction forces. Phenylephrine hydrochloride, 5 mg intramuscularly, decreases ballistic amplitude and heart rate but increases arterial pressure and high frequency components. Parallel change in arterial pressure and the magnitude of high frequency early diastolic ballistic forces suggest that aortic valve closure contributes to the genesis of the diastolic complex. Since ballistic amplitude and Fourier spectrum may vary independently, harmonic analysis may prove clinically useful as a diagnostic criterion.

Evidence from this and other laboratories indicates that the ballistocardiogram is largely the result of acceleration of a central arterial volume(3). Through the courtesy of Dr. E. Wetterer, we obtained canine aortic velocity pulses recorded with an electromagnetic flowmeter. Fig. 2 compares the frequency spectrum of such a pulse and of its time derivative with spectra of human velocity and acceleration ballistocardiograms. This is justified because central flow pulses and ultra-low-frequency ballistocardiograms are morphologically comparable in dog and man(5).

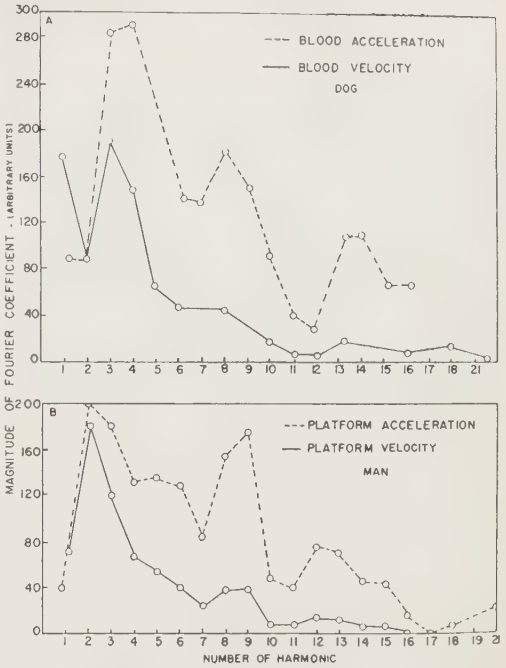

FIG. 2. Comparison between frequency spectra of canine blood flow velocity and acceleration pulses and human velocity and acceleration ballistocardiograms.

The striking similarity between the frequency spectrum of blood and platform motion supports the view that the ballistocardiogram is related to central blood flow.

Summary. Ballistocardiograms of the Starr and Nickerson type exhibit prominent resonance peaks, and contain no significant frequency components above 10 CPS. Ultra-low-frequency ballistocardiograms are distorted by body resonance to a far lesser degree, and exhibit components to at least 20 CPS. The band 0.5-20 CPS is therefore the minimum though not necessarily optimum frequency range for force ballistocardiography. The effects of respiration and vasoactive drugs suggest that harmonic analysis may prove useful as a diagnostic criterion for clinical ballistocardiography. Frequency spectra of blood velocity and acceleration pulses and of velocity and acceleration ballistocardiograms are strikingly similar, suggesting a cause and effect relationship between these wave forms.

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Localization of Thyroid and Spinal Cord Autoantibodies by Fluorescent Antibody Technic.* (23855)

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Recently production of autoantibodies against constituent(s) of the thyroid gland, presumably thyroglobulin, has been reported from this laboratory(1-6). Highly specific autoantibodies have been observed in several experimental animal species, such as rabbits, dogs, and guinea pigs, as well as in human beings suffering from certain types of chronic thyroiditis. Concurrently, characteristic changes in thyroid gland were frequently found. Demonstration of paralysis by immunization of experimental animals with brain substance has created considerable interest(7-9). In many instances brain-specific circulating antibodies were found in serum of these animals by both complement fixation and precipitation tests. However, antibody formation against brain substance including that of antibody-producing animal has been noted in absence of observable paralysis. On the other hand, brain lesions have been observed repeatedly in absence of detectable circulating antibodies. Therefore, relationship of circulating antibodies to what is frequently referred to as *allergic encephalomyelitis* is still a moot question. The fluorescent antibody technic of Coons and Kaplan(10) seemed to offer an interesting approach to the study of the possible direct combination of circulating antibodies and tissue sections. This technic might shed some new light on specific localization of tissue-specific antibodies. Simultaneous studies were therefore undertaken of these 2 autoantibody systems with Coons technic since they seem to constitute perfect controls for each other.

Methods. Sections of quick frozen tissues were cut 6 to 8μ thick with standard microtome in a cryostat according to method of Coons and Kaplan. The air-dried sections were fixed in formalin or in formalin followed by acetone. Formalin fixation was carried out by immersion in 10% formalin in pH 7.2 buffered saline solution at room temperature. Thyroid sections could be fixed in formalin for 2 to 10 minutes. Formalin fixation followed by 10 min. acetone fixation also gave satisfactory results. However, 2-minute formalin fixation was used in most experiments. Brain and spinal cord sections, on the other hand, were fixed in formalin for only 2 minutes because they lost their capacity to stain by fluorescent antibody technic when fixed in formalin for longer than 2 minutes or when 2 minute formalin fixation was followed by acetone fixation. Rabbit antisera to homologous brain or spinal cord antigens were prepared by intracutaneous injection of 20% aqueous suspensions in equal volume of Freund adjuvant. Aqueous suspensions were prepared by homogenizing fresh brain or spinal cord in Servall omnimixer for 30 seconds at maximum speed and centrifuging 20 seconds. As source of thyroid autoantibodies anti-rabbit thyroid rabbit sera were used from the same series as in previous studies(3,4,5). Complement fixation tests for thyroid autoantibodies and spinal cord autoantibodies as well as tanned cell hemagglutination tests with experimental thyroiditis sera were carried out as described previously(1,11). Evaluations of histologic

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FIG. 1. Photomicrograph of section of normal rabbit spinal cord (white matter) treated with rabbit thyroid autoantiserum (R 515) and stained with fluorescein isocyanate conjugate of goat anti-rabbit globulin antiserum. Only the blue autofluorescence of fixed spinal cord tissue is apparent (negative staining reaction).

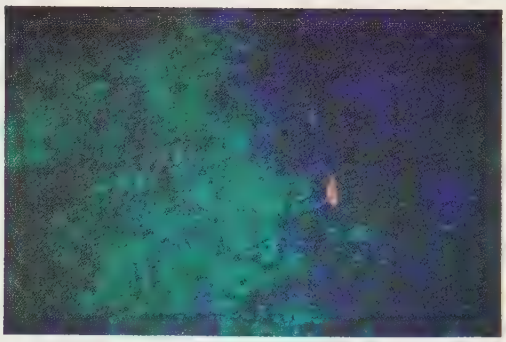

FIG. 2. Photomicrograph of section of normal rabbit spinal cord (white matter) treated with rabbit spinal cord autoantiserum (R 59) and stained as in Fig. 1. There is considerable amount of green fluorescence due to antibody-bound fluorescein; localization of green fluorescence corresponds to site of antigen-antibody reaction. Sites of reaction are suggestive of myelin sheaths (positive staining reaction).

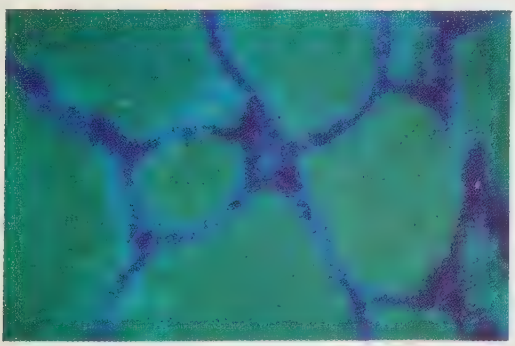

FIG. 3. Photomicrograph of section of normal rabbit thyroid treated with rabbit thyroid autoantiserum (R 515) and stained as for Fig. 1. Green staining of colloid indicates site of antigen-antibody reaction (positive staining reaction).

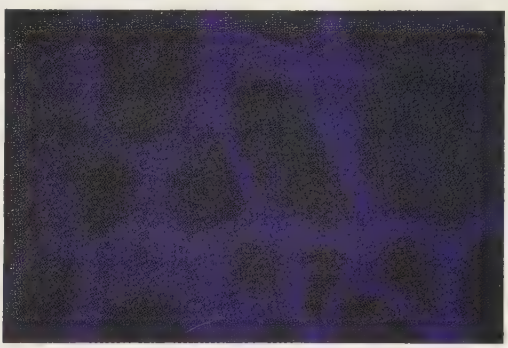

FIG. 4. Photomicrograph of section of normal rabbit thyroid treated with normal rabbit serum and stained as for Fig. 1. Only the blue autofluorescence of cellular elements is visible (negative staining reaction). The same result was obtained with rabbit thyroid sections treated with rabbit spinal cord autoantiserum (R 59).

All photomicrographs were made with dark field microscope and ultraviolet light source. Final magnification is 300 ×. We are indebted to Dr. J. Vazquez of University of Pittsburgh for taking the photographs.

changes of the thyroid gland were made as described previously(4). Observations for paralysis of rabbits immunized with homologous spinal cord or brain extracts were made daily. Fluorescent antibody staining was carried out by a two-step reaction; *i.e.*, sections of rabbit brain or spinal cord and rabbit thyroid were treated 30 to 60 min. with undiluted, active rabbit sera or serum dilutions, washed with buffered saline for 10 min., and stained with fluorescein isocyanate conjugated goat antiserum to rabbit immune globulin for 15 to 30 min., washed again and mounted. Goat antiserum was obtained from goats immunized with washed immune precipitates formed by reaction of goat serum and rabbit antisera to goat globulin. Goat antiserum was fractionated by precipitation with 50% saturated ammonium sulfate and the globulin fraction conjugated with fluorescein isocyanate† according to the method of Coons and Kaplan(10). The conjugated goat antisera to rabbit immune globulin were dialyzed against pH 7.2 buffered saline and adsorbed once or twice with acetone powders of rabbit or human livers as indicated by observed staining reactions. Some experiments were also carried out on rabbit and human thyroids by one-step staining reactions, *i.e.* rabbit and human thyroiditis sera were fractionated by 50% ammonium sulfate precipitation and the resulting globulin fraction was directly conjugated with fluorescein isocyanate. These conjugates were dialyzed and adsorbed with an ion exchange resin.‡ A few 2-step staining reactions were also carried out with human thyroid sections using human thyroiditis sera and rabbit antisera to human gamma globulin conjugated with fluorescein isocyanate. Observations of preparations were carried out with standard Reichert microscope using Reichert ultra-high intensity light source. Ultraviolet light was supplied by Osram 200 mercury vapor light. A UV pass filter transmitting light at 365 mμ was inserted between light source and specimen. The use of this filter made it possible to visualize autofluorescence of the fixed rabbit tissue as a blue color which contrasted clearly with the green color of fluorescein-labeled conjugates. A dark field condenser was used throughout. Fluorescent antibody staining reactions were regarded as negative when the intensity of reaction was no higher than that obtained with normal rabbit sera constantly used as controls. The colored photomicrographs of this publication were kindly made by Dr. Jacinto Vazquez of the University of Pittsburgh School of Medicine. The methods employed in making these photomicrographs have been described by Vazquez and Dixon(12).

TABLE I. Summary of Serologic Reactions of Rabbit Spinal Cord Autoantibodies with Homologous Antigens and Observations on Degree of Paralysis.

	Serologic reactions		
Rabbit serum*	Fluorescent antibody reactions	Complement fixation titers†	Degree of paralysis
59	Positive	320	++++
13	Questionable‡	80	none
14	Negative	40	++++
64	"	40	none

* All sera from rabbits immunized with rabbit spinal cord suspension with adjuvants.

† Greatest antiserum dilution giving complete inhibition of hemolysis using a 1:5 dilution of 20% rabbit brain.

‡ Usually weak positive reaction.

Results. Results of fluorescent antibody staining reactions of rabbit spinal cord sections obtained with 4 sera from rabbits immunized with rabbit spinal cord suspensions are summarized in Table I together with complement fixation titers of these sera and observations on presence or absence of paralysis. Definite positive staining reactions were consistently obtained with one of the 4 antisera. The results with a second serum were not quite as intense or consistent and were therefore interpreted as questionable. Staining reactions with 2 other sera were interpreted as negative. Interestingly enough, there appears to be a correlation between staining reaction and complement fixation titer. However, there appeared to be no correlation between serologic tests and presence of paralysis. Strongly positive staining reactions and complement

† We are indebted to Dr. Coons for synthesis of the fluorescein isocyanate. The fluorescein amine from which the isocyanate was prepared was donated by Sterling Winthrop Corp.

‡ Dowex 2-X, 200 mesh used according to method of Coons (unpublished).

TABLE II. Summary of *In Vitro* Reactions of Rabbit Thyroid Autoantibodies with Rabbit Thyroid Antigens and Observed Degree of Thyroiditis.

Sera	*In vitro* reactions: Fluorescent antibody reaction	*In vitro* reactions: Tanned cell hemagglutination titers	*In vitro* reactions: Complement fixation test	Degree of thyroiditis
515	Positive*	2560	Pos.	T.X.‡
558	" *	1280	"	Considerable
521	" †	320	Neg.	T.X.
555	Negative	81	"	Questionable
561	"	0	"	Neg.

* Positive staining reactions with serum dilutions up to 1:9.
† *Idem* 1:3.
‡ T.X. = Thyroidectomized. Rabbits' own thyroids were used as source of antigen for immunization.

fixation tests were obtained with 2 rabbit antisera to beef brain, using sections of rabbit brain or rabbit spinal cord as test material. Neither of the rabbits showed signs of paralysis.

Results of staining reactions with rabbit thyroid autoantisera are summarized in Table II. Of 5 rabbit sera described 3 with highest thyroid autoantibody titers regularly gave positive staining. Therefore, 2 systems of experimentally producible autoantibody tissue reactions are available, namely, thyroid autoantibody system and brain autoantibody system, which may serve as useful specificity controls for each other. Results of comparative experiments carried out to test specificity of systems are summarized in Table III. Specificity of autoantibody reactions for their homologous tissue is evident. Sera containing thyroid autoantibodies stain only the thyroid and not the spinal cord. In contrast, serum containing spinal cord autoantibodies stain only sections of spinal cord and not the thyroid.

Typical examples of staining reactions observed are shown in Fig. 1 to 4. Details of localization of staining reaction of rabbit spinal cord sections will have to be subjected to further scrutiny. However, as far as white matter is concerned, the axons appear as oval or round, dark, unstained areas surrounded or partially surrounded by brightly stained myelin sheaths. Such structures are seen in Fig. 1 in several places. No greenish staining was observed in spinal cord sections treated with rabbit thyroid autoantiserum or with normal rabbit sera.

Rabbit thyroid sections treated with selected thyroid autoantisera were intensely stained. Most of this staining is localized in the colloid. (Fig. 4). A thin rim of epithelium is also usually brightly stained. This is readily discernible in sections from which the colloid has been removed accidentally or intentionally by mechanical manipulation in the cryostat. Stroma of thyroid appears essentially unstained. Rabbit thyroid sections treated with antisera against rabbit spinal cord or with normal rabbit sera do not exhibit positive staining reactions.

The staining reactions referred to so far were all obtained by the 2-step method. Essentially, this method consists of adding rabbit sera containing autoantibody to sections of their homologous tissue as a first step, followed by addition of fluorescein-labeled goat antiserum containing antibody against rabbit immune globulin. Further experiments were carried out to obtain fluorescent staining by a one-step procedure. To this end a rabbit serum containing thyroid autoantibodies was fractionated and conjugated, as described pre-

TABLE III. Test of Specificity of Fluorescent Antibody Staining of Rabbit Thyroid and Rabbit Spinal Cord with Autoantibodies.

Auto-antiserum		Tissue sections: Rabbit thyroid	Tissue sections: Rabbit spinal cord
Thyroid	515	Positive	Negative*
	521	"	"
	558	"	"
Spinal cord	59	Negative	Positive

* Negative reactions are defined as those which are indistinguishable from the ones obtained with normal rabbit serum.

viously, and added to sections of normal rabbit thyroid glands. (This conjugate was adsorbed with ion exchange resin only to remove unconjugated fluorescein derivatives.) Definite characteristic staining was observed, comparable to that observed using the 2-step reaction.

Staining reactions similar to those in experimental rabbit thyroiditis have been obtained with sections of normal human thyroids using sera from patients with chronic thyroiditis and a conjugated rabbit antiserum to human gamma globulin for second step of the reaction.

Discussion. The organ-specific nature of brain antigen was first observed by Brandt, Guth and Müller(13) using alcoholic brain extracts as antigens. Witebsky and Steinfeld (14,15) studied appearance of brain-specific antibodies in rabbits following injection of aqueous brain suspensions of foreign species. The autoantibody nature of these brain hetero-antibodies was demonstrated by the reaction of rabbit antisera with rabbit brain suspensions and extracts. Rivers and Schwentker(7) demonstrated appearance of paralysis in monkeys following prolonged immunization with aqueous emulsions and alcohol ether extracts of rabbit brain. Appearance of paralysis following immunization with brain extracts in Freund's adjuvant was demonstrated by Kabat and co-workers(8) and Morgan(9).

The relation of circulating antibodies to appearance of characteristic lesions in the central nervous system is still an unanswered question. The problem at present is whether circulating antibodies combine with brain matter *in vivo* or are just incidental findings. A similar problem presents itself in the case of chronic thyroiditis both experimentally produced in animals(1-5) as well as in patients suffering from this disease(3-6).

The Coons technic(10) allowing recognition of antibodies fixed to tissue cells seems to offer interesting possibilities for the study of localization and fixation of antibodies in certain tissues and even within certain parts of specific cells involved. White(16,17) reported staining reactions obtained with human thyroid tissue and serum of patients with thyroid antibodies. At the same time one of us (EW)(16) reported staining reactions obtained with rabbit thyroid sections and sera of rabbits immunized with rabbit thyroid suspensions. Hiramoto, Engle and Pressman (18) report staining reactions obtained with human thyroid tissue and serum of a patient with thyroid antibodies, using rhodamine conjugation method developed by them.

To determine whether positive results obtained by our fluorescent antibody technic, are really specific in nature and not due to non-specific adsorption of antibody globulins by themselves, comparison of the 2 autoantibody systems with completely different specificities seemed to constitute excellent mutual controls. The results here reported demonstrate the feasibility of application of Coons technic to specific combination of autoantibodies and their corresponding tissue constituents.

Summary. (1) Sections of normal spinal cords of rabbits were treated with rabbit antisera produced by immunization of rabbits with rabbit spinal cord suspensions; the treated sections were stained with fluorescein conjugated goat antiserum to rabbit immune globulin. Selected areas stained suggest the myelin sheath as at least one of the antibody combining sites, though further histological studies are needed. No staining was obtained with normal sera or with rabbit sera containing thyroid autoantibodies. (2) In the same manner sections of thyroid glands of normal rabbits were exposed to the action of rabbit sera containing rabbit thyroid autoantibodies and stained with fluorescein conjugated goat antiserum to rabbit immune globulin. Positive staining reactions were obtained in the colloid extending into adjoining epithelial layer. Interstitial tissue remained basically unstained. No positive staining reactions were obtained using normal rabbit serum or rabbit serum containing brain autoantibodies. (3) With thyroid autoantibodies, the positive staining reactions and antibody titers seemed to run roughly parallel. However, rabbit autoantisera failed to give staining reactions when diluted above ninefold. On the basis of limited experience with spinal cord autoantisera, it is not possible to make a state-

ment regarding appearance of positive staining reactions and presence of demonstrable circulating antibodies, though the data obtained are suggestive of a correlation.

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